# Extraocular photoreception and colour plasticity in caterpillars of the peppered moth, *Biston betularia*


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# Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or processional qualification except as specified.

Chapter 2 of this thesis has been published in [Eacock, A., Rowland, H.M., Edmonds, N., Saccheri, I.J. (2017). Colour change of twig-mimicking peppered moth larvae is a continuous reaction norm that increases camouflage against avian predators. *PeerJ*, **5**, e3999]. For this publication, raw data for the experiments, 'Response to luminance gradient' and 'Response to heterogeneous colour environments' were collected by N. Edmonds and analysed by myself.

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# Abstract

Visual camouflage is a textbook example of natural selection, and a widespread strategy used by both predators and prey to avoid detection. Background matching, where the animal resembles the colour, brightness, and/ or pattern of the surrounding visual background is a common form of visual camouflage, and can occur through genetic polymorphism, behavioural background choice, or dynamic colour change. Dynamic colour change can occur very rapidly (milliseconds) or gradually, sometimes taking weeks to complete. Visual cues such as colour, brightness, and pattern, have been shown to elicit colour change, and in some colour-changing animals visual cues are sensed outside of the eye using extraocular photoreceptors (EOPs). Colour change research has been focused predominantly on rapid, chromatophore-based colour change, as observed in cephalopods. In contrast, little is known about the physiology and evolutionary origins of gradual colour change.

To avoid predation in a wide range of environments, caterpillars of the peppered moth (*Biston betularia*) masquerade as twigs and gradually change colour to match them. This thesis investigates the colour-changing response in *B. betularia* larvae: the shape of the reaction norm to colour and brightness gradients; the use and molecular basis of extraocular photoreception; and whether *B. betularia* alter resting behaviour to maximise concealment. Through a series of artificial twig experiments, I found that *B. betularia* larvae respond to both colour and luminance cues to produce a continuous range of phenotypes, rather than being restricted to a brown/green polyphenism as previously reported. To test for the possibility of extraocular photoreception, I occluded the eyes (ocelli) of groups of larvae and compared responses to colour and luminance with non-blindfolded control larvae. There was no difference in the colour-changing response of blindfolded larvae compared to controls, and blindfolded larvae also rested on colours that better matched their own colour to the same extent as non-blindfolded controls. I next examined the potential for visual machinery in the larval dermis, finding expression of a suite of visual genes throughout dermal tissue in *B. betularia* larvae and adults. In larvae, this expression was generally much higher relative to head tissue than found for adults. This finding corroborates the morphological and behavioural evidence for dermal photoreceptors in *B. betularia* larvae.

The final chapter is an attempt to examine the exclusivity of extraocular photoreception in *B. betularia*, and its evolutionary origins, through tissue-specific measurement of opsin expression in larvae and adults of a phylogenetically broad sample of Lepidoptera. Dermal opsin expression was found in other species, but depended on the gene (UV, blue, LW1, LW2) and developmental stage. Phylogenetic signal was found only for expression of LW1 in larvae, and LW2 in adults. Larval colouration strategy between species also appears to affect dermal opsin expression.

The thesis provides strong evidence for a novel physiological phenomenon: extraocular colour photoreception in the dermis of an insect, used to mediate colour change and behavioural background choice. The observation that dermal opsin expression occurs in several other species suggests that EOPs may be widespread in the Lepidoptera. Future work should be directed at the challenging task of understanding the mechanism underlying this class of EOPs, and characterising their functional roles in other species.

# Table of contents

## Chapter 1. General Introduction



## Chapter 2. Colour plasticity in Biston betularia: the reaction norm



## Chapter 3. Extraocular photoreception: colour response of blindfolded caterpillars

## Chapter 4. Behavioural background matching and extraocular photoreception in Biston betularia larvae



## Chapter 5. Mechanism of photoreception in Biston betularia: visual genes and electrophysiology



## Chapter 6. Extraocular opsin expression in Lepidoptera

## Chapter 7. General discussion



## Supplementary information

# Chapter 1
## General Introduction

### CAMOUFLAGE

Camouflage is a common anti-predator strategy that has resulted in some of the most diverse phenotypes seen in nature, driven by the selective pressure of visual hunters (Stevens & Merilaita, 2009). Indeed, the first theories of evolution through natural selection were stimulated by examples of camouflage (Wallace, 1879). Camouflage has since been a popular topic of research interest, resulting in identification of numerous types of camouflage in a wide range of systems. However, there are still major knowledge gaps, particularly with regard to the mechanisms and adaptive value of colour change across species. In many studies, camouflage has not been measured from the perspective of the appropriate predator, which makes it difficult to infer its ecological-evolutionary significance.

There are several ways in which animals achieve visual camouflage, including distractive markings, disruptive, or dazzle colouration, countershading, and masquerade (see Table 1.1 for definitions and examples). Arguably, one of the most common forms of camouflage is background matching, where an animal resembles the colour, brightness, and/or pattern of their surrounding environment (Cott, 1940; Endler, 1981), to avoid detection (Merilaita *et al.*, 2001; Merilaita & Dimitrova, 2014).

**Table 1.1.** Summary of different categories of visual camouflage

| Camouflage type | Definition | Examples |
|---|---|---|
| Distractive markings | These markings direct the 'attention' or gaze of the receiver from traits that would give away the animal's outline and therefore the animal (Stevens & Merilaita, 2009). | • Bright markings on wings of Lepidoptera such as the comma butterfly, *Polygonia c-album* and the silver Y moth, *Autographa gamma* (Dimitrova *et al.*, 2009);<br>• The white tail tips frequently found in carnivorous mammalian grassland predators (Caro, 2011). |
| Disruptive colouration | Markings that create the appearance of false edges and boundaries, therefore hindering the detection or recognition of an object's true outline and shape (Cott, 1940). | • Dynamic patterning in cephalopods (Hanlon *et al.*, 2009);<br>• Markings in water snakes (Beatson, 1976);<br>• Marine isopod (Merilaita, 1998). |
| Motion dazzle | Markings that inhibit the predator's judgment of the speed and trajectory of a moving prey animal (Thayer, 1909). | • Zebra (How & Zanker, 2014);<br>• Cuttlefish (Zylinski *et al.*, 2009). |
| Countershading | Darker colouration on the dorsal side than on the ventral side. This cancels out the dorsoventral illumination gradient across the body, thus eliminating the outline, enhancing background matching (Thayer, 1896). | • Primates (Kamilar, 2009);<br>• Deer, and other species of ruminant (Allen *et al.*, 2012);<br>• Lepidopteran larvae (Rowland *et al.*, 2008);<br>• Coral reef fish (Hamilton & Peterman, 1971). |
| Masquerade | Instead of avoiding detection, the animal mimics an uninteresting or inedible object in their environment, causing predators to misclassify it (Skelhorn *et al.*, 2010a). | • Lepidopteran larvae masquerading as twigs (Skelhorn *et al.*, 2010c), or bird faeces (Suzuki & Sakurai, 2015);<br>• Stone-mimicking succulent plants (Lev-Yadun, 2014);<br>• Leaf masquerade in spiders (Kuntner *et al.*, 2016), and *Kallima* butterflies (Suzuki *et al.*, 2014);<br>• Snail masquerade in juvenile cichlids (Satoh *et al.*, 2017). |
| Background matching/ cryptic resemblance | When the animal avoids detection by resembling the colour, brightness, and/ or patterning of the visual background (Cott, 1940). | • Stick insects (Sandoval, 1994a);<br>• Geometrid moths (Kang *et al.*, 2012);<br>• Tree frogs (Wente & Phillips, 2003);<br>• Snowshoe hare (Mills *et al.*, 2013). |

Background resemblance can be achieved through allele (morph) frequency change between generations; through habitat choice within generations; and through plastic (physiological or developmental) responses by the same individual (Stevens, 2016). Cryptic phenotypes maintained through visually hunting predators exist in numerous animal taxa including arthropods, fish, birds, reptiles, and mammals (Ryer *et al.*, 2008; Pellissier *et al.*, 2011; Olsson *et al.*, 2013; Surmacki *et al.*, 2013; da Silva *et al.*, 2016; Stoddard *et al.*, 2016), where the molecular basis for many of these

adaptations is well known (Rosenblum *et al.*, 2004; Corso *et al.*, 2012; van't Hof *et al.*, 2016). These fixed polymorphisms successfully defend against predation in a fixed habitat type (Merilaita & Dimitrova, 2014; Troscianko *et al.*, 2016), but do not provide flexibility when animals are faced with environments that change over time or are heterogeneous in appearance among patches in space. The most famous example of phenotype-environment mismatching is industrial melanism in adult peppered moths (*Biston betularia*), where pale and dark morphs mismatched against soot polluted and 'clean' trees, respectively, results in relatively higher risk of predation (Cook *et al.*, 2012). Colour polymorphic *Timema cristinae* stick insects are locally adapted to different host plants, where increased predation also occurs on maladapted individuals (Farkas *et al.*, 2015).

Animals with fixed phenotypes can improve their camouflage through behaviour. Many fish and insect species choose to rest on backgrounds that best match their own colour or pattern (Calver & Bradley, 1991; Kjernsmo & Merilaita, 2012; Tyrie *et al.*, 2015), or orient themselves to improve crypsis against their surroundings (Kang *et al.*, 2012). In salamanders, the different resting behaviours of two colour morphs are thought to contribute to the maintenance of the colour polymorphism itself (Venesky & Anthony, 2007).

**PLASTIC COLOUR CHANGE**

For organisms living in environments that are heterogeneous over space and time, adaptation may favour phenotypic plasticity (Agrawal, 2001). Phenotypic plasticity is the ability of an organism with a single genotype to express different phenotypes in response to biotic or abiotic environmental factors (Travis, 1994). Plastic phenotypes exist for a number of reasons (Gotthard & Nylin, 1995), including thermoregulation (Trullas *et al.*, 2007), social signalling (Barbato *et al.*, 2007; Stuart-Fox & Moussalli, 2008), predator avoidance (Tollrian, 1995; Frommen *et al.*, 2011; Heynen *et al.*, 2017), and camouflage (Stuart-Fox *et al.*, 2008). Animals that can change colour in response to a rapidly changing visual environment gain additional benefits through crypsis, which has been observed in a wide range of taxa, such as arthropods, fish, cephalopods, and mammals (Mills *et al.*, 2013; de Bruyn & Gosselin, 2014; Stevens *et al.*, 2014a; Gilby *et al.*, 2015; Stevens *et al.*, 2015; Duarte *et al.*, 2016). This ability is particularly important for the survival of species with high dispersal and vulnerable larval stages, which might find themselves in rapidly changing and unpredictable environments (Stevens, 2016).

Developmental plasticity often occurs to protect vulnerable life stages from predation, as seen in some benthic invertebrates, which change colour to maximise concealment in different coloured backgrounds (de Bruyn & Gosselin, 2014). Larvae of the swallowtail butterfly masquerade as bird droppings during early instars, later becoming green in colour, until final instar (Futahashi & Fujiwara, 2008b). Similar phenotypic switches have been reported in other species of Lepidoptera; for example, larvae of the genus *Nemoria*, switch between oak catkin mimics in the spring and oak branches in the summer (Greene, 1989). Cotton bollworm (*Helicoverpa armigera*) larvae change colour and pattern into four main phenotypes: plain green, patterned green, brown, and black (Yamasaki *et al.*, 2009). In *Nemoria* and *Helicoverpa*, dietary cues are thought to be responsible for the switch between phenotypes (Greene, 1989; Yamasaki *et al.*, 2009), but visual cues could not be completely ruled out. An ontogenetic switch between discrete phenotypes is known as a polyphenism (Mayr, 1963).

Polyphenism may be beneficial for animals moving between patch types that do not vary much across time and space, but do not provide as much flexibility as phenotypes that can change on a continuous scale. A plastic trait that can be measured on a continuous scale is a reaction norm (Woltereck, 1909). Arthrophods, such as crab spiders, sand fleas and crabs, as well as cephalopods, can change colour on a continuous scale (Mathger & Hanlon, 2007; Llandres *et al.*, 2013; Stevens *et al.*, 2014b; Stevens *et al.*, 2015). There is only one study reporting continuous colour change in Lepidoptera, where larvae of two species of hawk moth, *Laothoe populi* and *Smerinthus ocellata*, changed colour depending on the wavelength of their rearing background (Grayson & Edmunds, 1989).

## PHYSIOLOGY OF COLOUR CHANGE

Colour change may be divided into two broad types, based on the underlying mechanism: *physiological* or *morphological*, which differ both in the rate of colour change and how it is produced. Physiological colour change is rapid, and can occur in milliseconds (Mathger *et al.*, 2003), and is achieved through movement of pigment in specialised cells known as chromatophores, of which there are three types: xanthophores, iridophores, and melanophores (Bagnara *et al.*, 1968). Xanthophores contain a yellow pigment and are the outermost component. Underneath the xanthophores, the light-reflecting iridophores are located, which give a shiny appearance. Melanophores are found underneath the xanthophores, which

contain the dark pigment, melanin (Bagnara *et al.*, 1968). Muscle contraction of these three layers allows for the impressive and extensive visual displays of animals like squid, cuttlefish, chameleons and fish (Mathger *et al.*, 2003; Mathger & Hanlon, 2007; Stuart-Fox & Moussalli, 2009).

In contrast, morphological colour change occurs slowly, taking days to months to complete, through gradual deposition of pigments in the dermis (Oshima, 2001). Colour change has been investigated for over 100 years, but most of the research effort has been directed towards understanding physiological colour change (Stevens, 2016). There are very few studies on the mechanism of morphological colour change, crab spiders (*Misumena vatia*) being one of the best-described examples. *Misumena vatia* change colour between white and yellow, over several days to match the colour of the flowers on which they rest (Morse, 2007). The pigments involved in this colour change have been identified as ommochromes, which are also responsible for the dermal colours of other arthropods, including stick insects and locusts (Linzen, 1974; Buckmann, 1977). Behavioural, electrophysiological, and morphological data show that visual cues elicit colour change in *M. vatia* (Insausti & Casas, 2008; Insausti *et al.*, 2012). Visual cues have also been implicated in colour change of lepidopteran larvae and pupae (Poulton, 1892; Grayson & Edmunds, 1989; Noor *et al.*, 2008), but so far this evidence has been somewhat inconclusive, as the contribution of chemical or tactile cues in addition to visual cues, is uncertain.

**VISUAL PERCEPTION**

The vast majority of animals depend on visual perception to orientate themselves in their environment, and to perform complex behaviours such as dispersal, foraging, mating, and predator avoidance, including camouflage. Different animals perceive brightness, colour, and motion differently, due to alternative selective forces and phylogenetic constraints that have shaped visual systems. Therefore, colour is not an inherent property of an object; it is defined by the sensory system of the viewer (Endler, 1990; Guilford & Dawkins, 1991).Vertebrate vision and phototransduction have been studied in greater detail than invertebrates, particularly in the context of human medicine (Kaupp & Koch, 1986; Palczewski, 2000). Currently, the *Drosophila* visual system provides the best information on insect phototransduction (Hardie, 1996; Montell, 1999; Hardie & Raghu, 2001), which likely does not accurately represent all aspects of the structure and functioning of visual perception in other insect groups.

**Molecular basis of phototransduction**

In most vertebrates, phototransduction occurs in the eye, where photoreceptive cells are located, known as rods and cones (Hecht, 1937). There are major functional differences between rods and cones. Rods are primarily involved in achromatic vision and are capable of detecting a single photon of light, making them extremely sensitive, even in dim light (Baylor *et al.*, 1979). In contrast, cones are 100-fold less sensitive than rods (Rushton, 1965), and are sensitive to colour. Cone cells contain visual pigments, which are G protein-coupled receptors, each consisting of an opsin protein bound covalently to a vitamin-A derived chromophore, 11-*cis*-retinal. Bound opsin and retinal are known as rhodopsin (Hargrave, 2001). Upon the absorption of a light photon, the chromophore is isomerised from 11-*cis*-retinal to all-*trans*-retinal, causing a conformational change in the opsin protein. Photo-activated opsin activates the G-protein, transducin, initiating the phototransduction cascade that results in a drop in cGMP concentration and hyperpolarisation of the plasma membrane and signalling of second order neurons (Fig. 1.1A). Following the cascade, opsin is then phosphorylated and bound to arrestin, preventing further activation by transducin (Hargrave, 2001). Another important gene in the phototransduction pathway is retinal degeneration B (RDB). The function of RDB in vertebrate vision is not yet fully understood, but it has been studied in invertebrates, where *Drosophila* RDB mutants have been found to display abnormal electrophysiological light responses and subsequent photoreceptor degeneration (Harris & Stark, 1977; Milligan *et al.*, 1997).

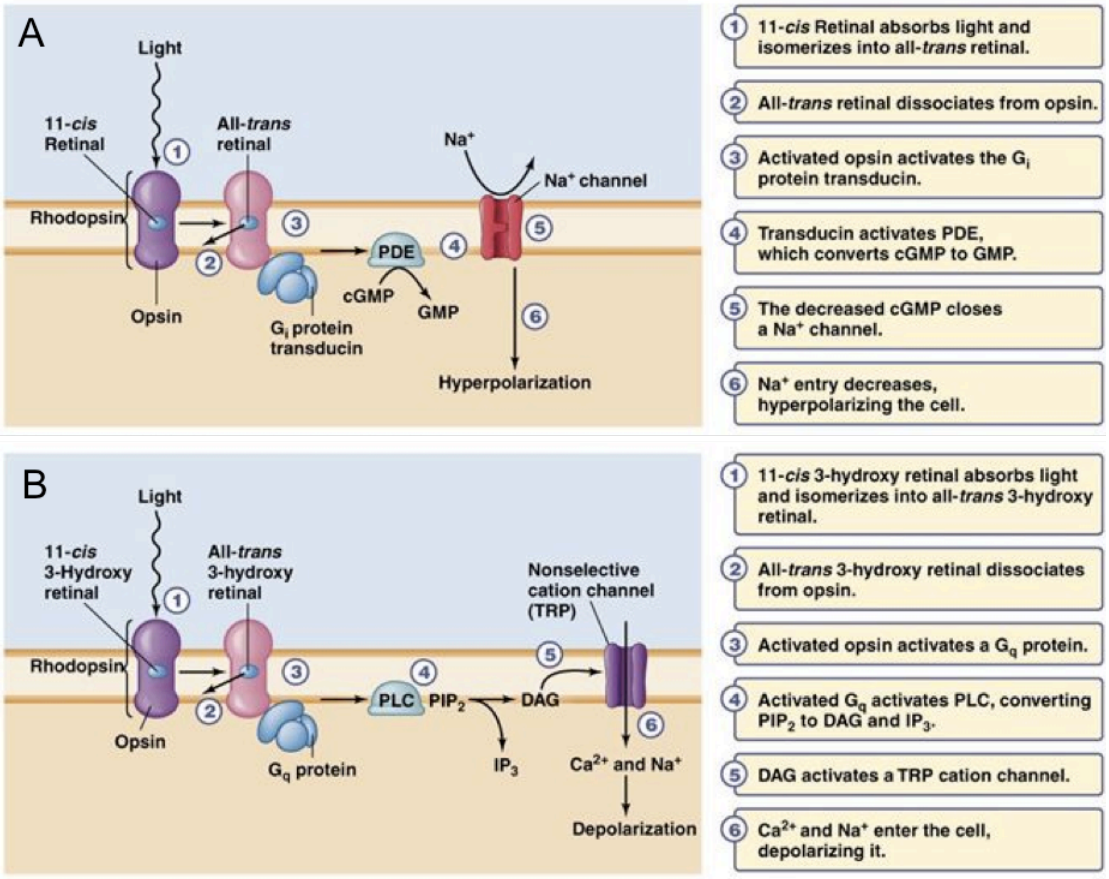


**Figure 1.1. Phototransduction pathways for (A) vertebrate and (B) invertebrate photoreceptors.** Images adapted from (Moyes & Schulte, 2007).

Phototransduction in invertebrates shares many similarities with vertebrate vision: both pathways are initiated by the interaction of rhodopsin and a G-protein (transducin), but in invertebrates, this interaction activates a different enzyme, phospholipase Cβ, which opens TRP and TRPL channels (Fig. 1.1B). This is the opposite of what occurs in vertebrates, where cGMP channels close, subsequent to a reduction in cGMP, caused by cGMP phosphodiesterase (Rayer *et al.*, 1990).

Invertebrate eyes are also structurally different to most vertebrate eyes, in that they are not comprised of rods and cones. The arthropod compound eye is instead made up of thousands of structures called ommatidia (Fig. 1.2B). Each ommatidium contains photoreceptor cells, arranged in a radial pattern (Nilsson *et al.*, 1988). The number of photoreceptor cells differs between species; for example, *Drosophila* possess eight photoreceptor cells per ommatidium in their compound eye (Ready *et al.*, 1976), whereas butterfly eyes contain nine photoreceptor cells (Briscoe, 2008). The larval stages of holometabolous insects, such as beetles, butterflies, moths, and flies have 'simple' eyes, with only 6 or 7 single ommatidia (Fig. 1.2A), usually

arranged in a semi-circle on each lateral side, known as stemmata or ocelli (Gilbert, 1994).

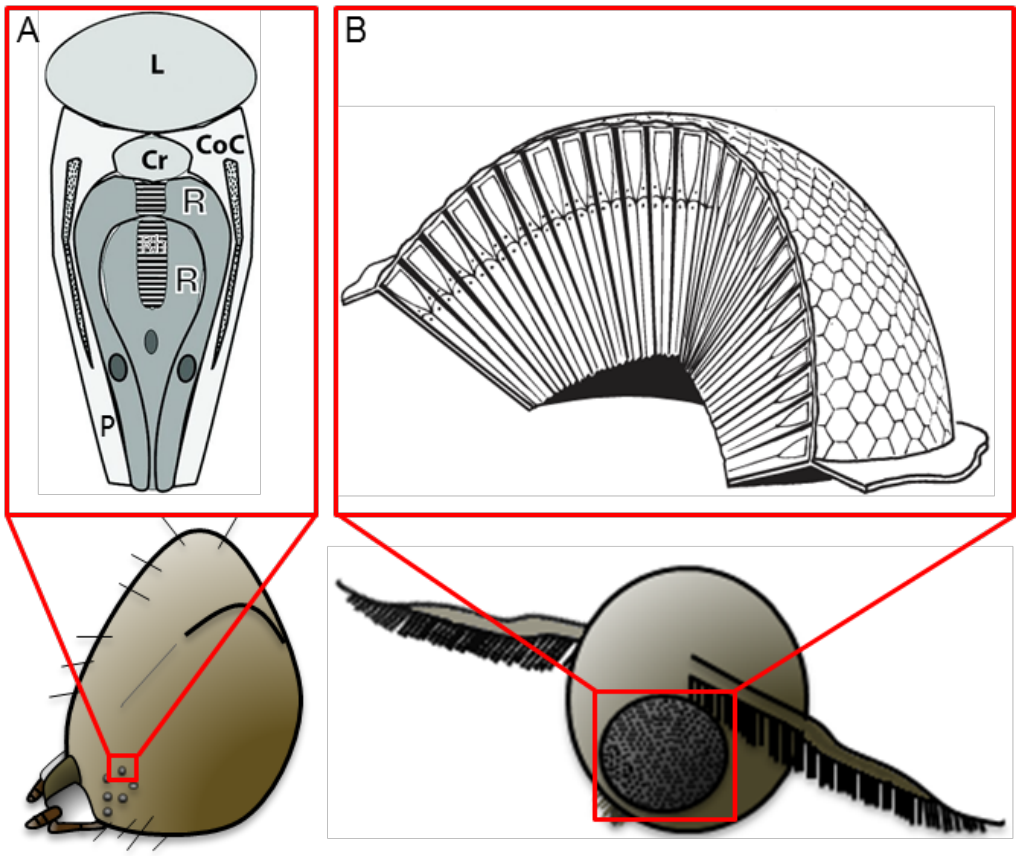


**Figure 1.2. Cellular organisation of larval and adult stage lepidopteran eyes.** (A) One of six ocelli situated on each side of head of larvae. L, lens; Cr, crystalline cone; CoC, corneagenous cell; R, retinula cells; Rh, rhabdom; P, pigment cells. (B) Compound eye comprised of numerous ommatidia; each individual ommatidium has an almost identical cellular organisation to each ocellus. Images adapted from drawings in Buschbeck (2014).

### Role of opsins in colour vision

Regardless of the differences observed in eye physiology and phototransduction pathways, opsin proteins are universal in their function of colour vision across the animal kingdom. Humans and closely related primates have trichromatic vision, facilitated by three types of cone cell, which differ in their response to three types of wavelength: short, medium, and long (Fig. 1.3). The different responses are determined by the likelihood that the opsin proteins will absorb photons of different wavelengths, i.e. how sensitive they are to each wavelength. Specific amino acid substitutions in opsin proteins create shifts in sensitivity to light wavelengths (Yokoyama, 2002).

Many fish and bird species are capable of tetrachromatic vision, with an additional cone to humans, enabling UV sensitivity. UV vision in these animals is thought to

aid mate selection and foraging (Jacobs, 1992; Bennett & Cuthill, 1994). The insect eye is also capable of vision into the UV range (Mazza *et al.*, 2002). Butterfly eyes in particular have a high diversity of opsin genes, associated with sexual selection and foraging for food and suitable host plants for oviposition (Wakakuwa *et al.*, 2010; Ogawa *et al.*, 2012). In addition to amino acid substitutions, opsin gene expression has also been found to affect the spectral sensitivity of visual pigments in fish (Hofmann & Carleton, 2009; Sakai *et al.*, 2016). In some animals, opsin expression is not restricted to the eye, and has been found in various dermal tissues (Okano *et al.*, 2000; Chen *et al.*, 2013).

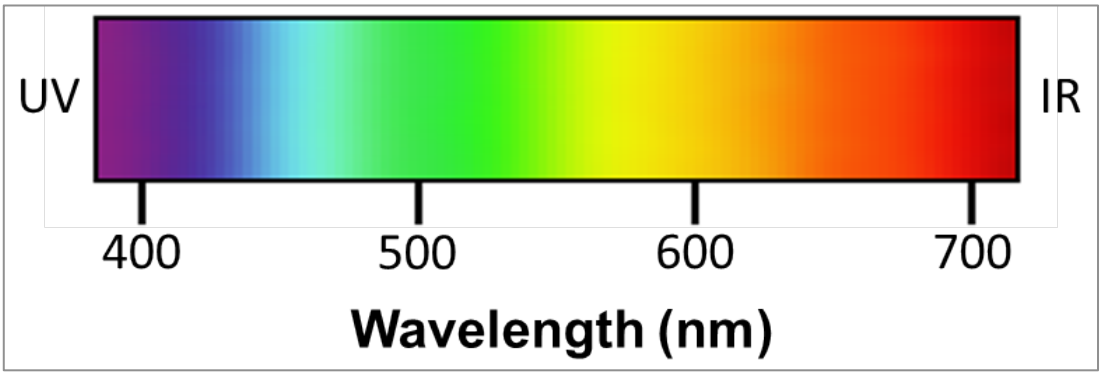


**Figure 1.3. Visible light spectrum.** Wavelength ranges for ultraviolet or UV (<400), violet (400-450), blue (450-500), green (500-570), yellow (570-590), amber (590-610), red (610-700), and infrared or IR (>700). Wavelengths range from short (UV) to long (IR).

## EXTRAOCULAR PHOTORECEPTION

Light sensing outside of the eye, or extra ocular photoreception (EOP) is known to occur in many animals, predominantly associated with circadian rhythm (Helfrich-Forster *et al.*, 2002; Ruby *et al.*, 2002; Fu *et al.*, 2005) but also other behaviours, including phototaxis, defense, and pigmentation (Ramirez *et al.*, 2011). Many mollusc and echinoderm species use dispersed dermal photoreceptors to withdraw when shaded (shadow response), and orientate themselves towards light (Millot, 1968; Jekely, 2009). There is further evidence for EOP controlled pigmentation in echinoderms (Millot, 1975), where the EOPs are likely to be located in chromatophore cells, forming a localised feedback mechanism between light perception and pigment dispersal/aggregation. EOPs have been linked to colour change in chromatophores of cephalopods, fish, lizards, and amphibians (Aspengren *et al.*, 2006; Chen *et al.*, 2013; Fulgione *et al.*, 2014; Kingston *et al.*, 2015).

Thus far, the behaviours known to be mediated by EOPs are simple light/dark responses and do not require image forming, or colour vision, with the exception of

chromatophore-based responses, which may be responses to colour, rather than luminance cues. As far as we are aware, only two examples of extraocular photoreception linked with gradual colour change exist; blindfolding experiments in lepidopteran pupae by Poulton, (1892) and Angersbach, (1975). Lepidopteran larvae and pupae are highly diverse in colour/ pattern, providing intriguing examples of mimicry and masquerade, many of which have not been explored from a mechanistic or evolutionary standpoint. Much more research is required to further understand extraocular photoreception in colour changing species.

**COLOUR CHANGE IN PEPPERED MOTH LARVAE**

Larvae of the peppered moth (*Biston betularia,* family: Geometridae) masquerade as twigs and change colour from green to brown to match them (Noor *et al.*, 2008; Skelhorn & Ruxton, 2010). They are initially wind dispersed and polyphagous (Tietz, 1972; Noor *et al.*, 2008), such that the range of twig colours they might encounter in nature is likely to be broad. Larvae typically undergo five moults, resulting in six instars until pupation, where larvae reach a final size of 5-6 cm in length. Each instar lasts around one week, except the final instar which can be up to two weeks for some individuals (Edmonds, 2010). In the first instar, larvae appear countershaded, and subsequently change colour, typically to brown or green, which has been described as a colour polyphenism (Noor *et al.*, 2008). Unlike for adult *B. betularia*, which display genetically fixed phenotypes (Cook *et al.*, 2012; van't Hof *et al.*, 2016), colouration the larvae is not under genetic control (Poulton, 1892; Noor *et al.*, 2008) and is not linked to the colour phenotypes observed in the adult moths (Noor *et al.*, 2008). However, both adaptations are thought to increase crypsis from bird predation. Blue tits (*Cyanistes caeruleus*) are known predators of Geometrid larvae (Arnold *et al.*, 2010). These factors make *B. betularia* a good model for studying colour change in the context of camouflage against avian predation.

The very first observations of colour change in *B. betularia* by Poulton (1892) and subsequent colour measurements by Noor *et al.* (2008) did not take the vision of predators into account. To understand the adaptive significance of colour change, calibrated stimuli are required, and colour needs to be measured from the perspective of the animal's natural predators (Stevens & Merilaita, 2009). As well as understanding the ultimate cause of camouflage, studies on colour change can provide knowledge on the proximate mechanisms involved; from the cues that elicit the response, to the hormones and pigments that result in the phenotypic change. In

general, most studies on morphological colour change have not identified the feedback mechanisms (i.e. dietary, visual) that control colour change, or the extent to which colour change improves camouflage and increases survival chances of the colour-changing organism.

Therefore, the purpose of the work in this thesis is to understand the proximate and ultimate mechanisms of morphological colour change, using *B. betularia* larvae as a model. The following questions are addressed in the subsequent data chapters:

1. **What is the extent of colour change in *Biston betularia*?**

   I tested whether larvae responded predominantly to luminance (brightness) or colour using artificial twigs, and whether the response is a phenotypic switch between green and brown, or if they can produce intermediate phenotypes. I modelled colour from the visual perspective of avian predators to provide evolutionary context.

2. **Are larvae using extraocular photoreceptors to perceive and match visual backgrounds?**

   I blindfolded larvae and reared them on different chromatic and achromatic visual stimuli to assess whether putative extraocular photoreceptors could differentiate colour from luminance.

3. **Does larval behaviour enhance camouflage through habitat selection?**

   I determined whether larvae rested more frequently on colours that provided a visual match to their own colour, and whether ocular photoreception is required to perform such behaviour.

4. **What are the visual capabilities of *Biston betularia*?**

   I tested the visual ability of ocular and extraocular photoreceptors in *B. betularia* larvae and imagines by quantifying visual gene expression. Additionally, I tested larval vision using electrophysiological techniques.

5. **What are the patterns of visual gene expression across the Lepidoptera?**

   I explored the phylogenetic distribution of extraocular opsin expression across a diverse sample of Lepidoptera and explored associations with particular life history traits, including colour plasticity. This will contribute towards understanding the evolutionary origins of extraocular colour perception in Lepidoptera.

# Chapter 2

## Colour plasticity in *Biston betularia*: the reaction norm

**ABSTRACT**

Camouflage, and in particular background matching, is one of the most common anti-predator strategies observed in nature. Animals can improve their match to the colour and/or pattern of their surroundings through background selection, and/or by plastic colour change. Colour change can occur rapidly (a few seconds), or it may be slow, taking hours to days. Many studies have explored the cues and mechanisms behind rapid colour change, but there is a considerable lack of information about slow colour change in the context of predation: the cues that initiate it, and the range of phenotypes that are produced. Here we show that peppered moth (*Biston betularia*) larvae respond to colour and luminance of the twigs they rest on, and exhibit a continuous reaction norm of phenotypes. When presented with a heterogeneous environment of mixed twig colours, individual larvae specialise crypsis towards one colour rather than developing an intermediate colour. Flexible colour change in this species has likely evolved in association with wind dispersal and polyphagy, which result in caterpillars settling and feeding in a diverse range of visual environments. This is the first example of visually induced slow colour change in Lepidoptera that has been objectively quantified and measured from the visual perspective of natural predators.

**INTRODUCTION**

Some of the most diverse and visually striking phenotypes seen in nature are those of camouflaged animals (Stevens & Merilaita, 2009). Background matching, or crypsis, is a common anti-predator strategy that has provided a test-bed for the theory of evolution through natural selection (Wallace, 1879; Wallace, 1889). Crypsis is selected for by visual predators such as birds (Merilaita *et al.*, 2001), whereby prey that match the colour/ pattern of the surrounding backgrounds survive for longer than non-matching prey (Endler, 1981; Merilaita *et al.*, 2017). In heterogeneous habitats, comprised of visually contrasting patches, or a gradient from one habitat type to another (Fig. 2.1), optimising crypsis on all of the background components presents a challenge (Merilaita *et al.*, 1999). One solution to this problem is a genetic polymorphism, which can produce two or more morphs that are specialised to different patch types (Merilaita *et al.*, 2001; Surmacki *et al.*, 2013). However, a species with a genetically fixed phenotype is restricted to camouflage on one background, or limited camouflage across varied patch colours (Fig. 2.1A). Therefore, in environments that change appearance across small temporal and spatial

scales, detrimental phenotype-environment mismatching can occur (Cook *et al.*, 2012; Farkas *et al.*, 2015). In this case, selection may favour phenotypic plasticity, enabling individuals to actively change their appearance to utilise different habitat patches without compromising camouflage (Fig. 2.1B; Stevens 2016). An example of plasticity is colour change, which is a topic of current research interest and can be used to study the adaptive value and the physiology of camouflage (Duarte *et al.*, 2017).

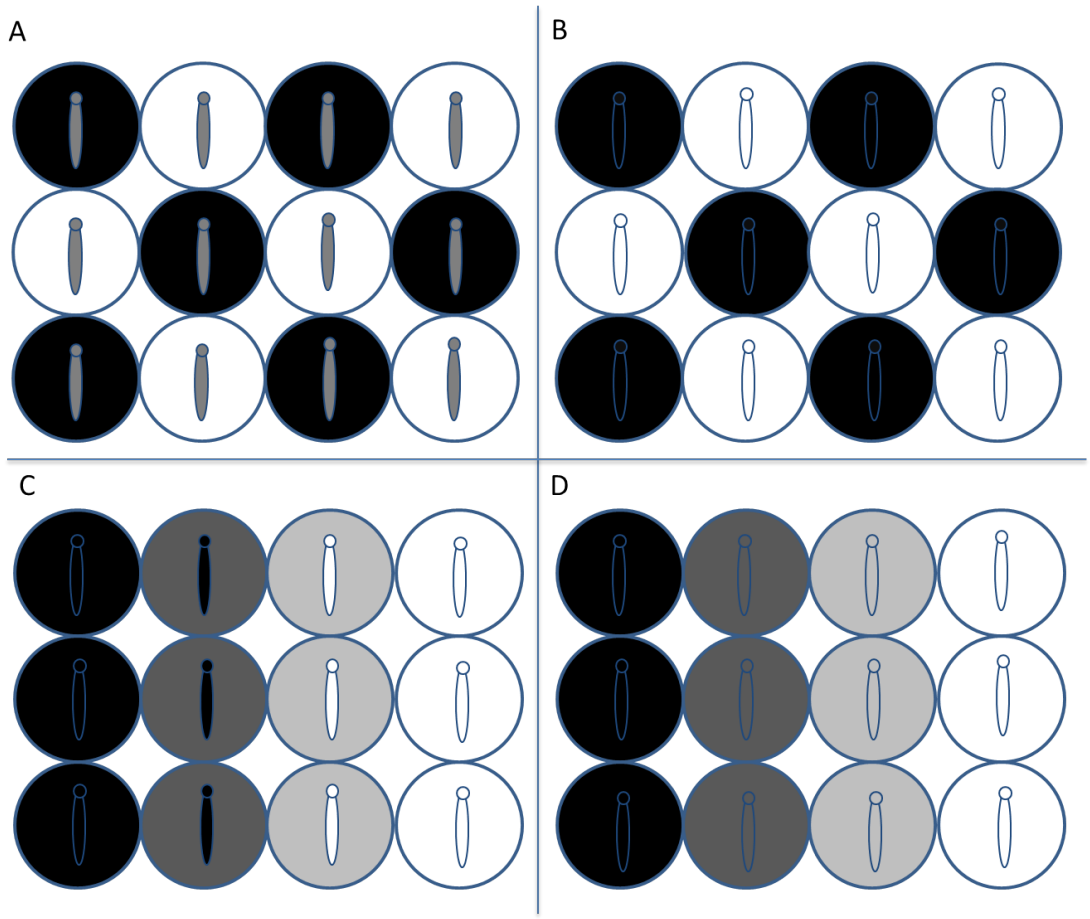


**Figure 2.1. Possible camouflage strategies of caterpillars in response to visually heterogeneous environments**. (A) In an environment composed of different coloured patches, caterpillars with a fixed genetic phenotype achieve compromised crypsis on all backgrounds. (B) The same habitat scenario as A, but with larvae specialised to match one patch type, either by genetic polymorphism, restricting individuals to one patch colour, or by plastic polyphenism, in principle allowing individual larvae to move between patches and switch colour to match their background. (C) Larvae with genetic polymorphism or plastic polyphenism inhabiting a graded environment with intermediate colour patches, where phenotypes match the extreme, but not the intermediate backgrounds. (D) An environmental gradient with intermediate backgrounds, where larvae produce a continuous colour response to background colour, allowing utilisation of each patch colour.

Rapid colour change (<2h), as reported in fish, cephalopods, and amphibians has been widely studied (Hanlon *et al.*, 2009; Buresch *et al.*, 2011; Allen *et al.*, 2015), and much is known about how chromatophores produce rapid changes in colour and

pattern in these systems (Mathger & Hanlon, 2007; Kingston *et al.*, 2015). Comparatively slower colour changes (days to months) occur in some arthropod and fish species (Ryer *et al.*, 2008; Llandres *et al.*, 2013). In many of these systems we still do not know whether slow colour change is adaptive, nor do we know the precise cues or biochemical processes involved. A number of potential cues have been proposed, with dietary and visual cues receiving most attention (Stevens & Merilaita, 2009; Duarte *et al.*, 2017).

One example of a diet-induced phenotypic switch, or polyphenism, is seen in the larval stage of the moth *Nemoria arizona*, which resembles inedible objects in its environment (Greene, 1989). In the spring the larvae resemble oak catkins, and in the summer they look like the branches of oak. This form of visual resemblance to inanimate objects is referred to as masquerade (Skelhorn *et al.*, 2010a). Masquerade enables prey to avoid attack because predators misclassify these prey, rather than failing to detect them (Skelhorn *et al.*, 2010c). The larvae of the peppered moth (*Biston betularia*) also masquerade as the twigs of their food plant and change colour to match them (Poulton, 1892; Noor *et al.*, 2008). These brown and green colour morphs occur in response to the background colour on which the larvae rest (Poulton, 1892; Noor *et al.*, 2008). Changing appearance in response to background cues in the environment may be beneficial for animals that masquerade, as masquerade is often associated with polyphagy (Higginson *et al.*, 2012). Visually hunting predators, like birds, heavily predate caterpillars that do not display warning colours (Lichter-Marck *et al.*, 2015), and twig-mimicking caterpillars that do not match the twigs they rest on are also more likely to be predated (Skelhorn & Ruxton, 2010). Therefore, the ability to change colour could enhance masquerade in the wider range of environments these prey are likely to encounter, and consequently reduce their foraging restrictions (Ruxton *et al.*, 2004).

It is important to determine the exact cues eliciting colour change, as these cues initiate the colour change cascade (Duarte *et al.*, 2017), and can therefore provide information on the evolution of adaptive colour and the mechanisms of colour production (Cuthill *et al.*, 2017). Visual stimuli exist in two forms: achromatic (luminance), and chromatic (hue/chroma). Responses to achromatic stimuli (luminance) have been reported in sand fleas, geckos, toads, and flatfish (Ryer *et al.*, 2008; Vroonen *et al.*, 2012; Stevens *et al.*, 2015; Polo-Cavia *et al.*, 2016). Tree frogs (*Hyla japonica*) adjust their body colour and luminance, to maximise camouflage

against visually heterogeneous backgrounds, although the response to achromatic stimuli was stronger (Choi & Jang, 2014; Kang *et al.*, 2016). Many of these studies propose that colour change in these animals is induced by visual cues, but the visual pathways were not explicitly studied, and additional cues such as temperature or texture were often not controlled for (Lin *et al.*, 2009; Yamasaki *et al.*, 2009; Polo-Cavia *et al.*, 2016).

To address this topic, we conducted a series of experiments to explore the type of visual cues that elicit colour change in *B. betularia*. The colour change in *B. betularia* has previously been described as a polyphenism: a switch of phenotype (Noor *et al.*, 2008). However, in the only study so far to investigate this behaviour, Noor et al (2008) only provided two discrete stimuli: green vs. brown, and measured colour subjectively from a human perspective. The larvae of *B. betularia* are polyphagous and wind dispersed as first instar larvae (Tietz, 1972; Noor *et al.*, 2008). The wide variety of twig colours between and within host plant species (Edmonds, 2010) presents a highly heterogeneous resting background. Therefore, it may be beneficial for individuals to change appearance on a continuous scale over time (Fig. 2.1D), known as a reaction norm (Woltereck, 1909). Colour reaction norms have been reported in squid, geckos, and anurans (Mathger & Hanlon, 2007; Vroonen *et al.*, 2012; Kang *et al.*, 2016), and are commonly induced by visual stimuli aquired by the animal about its environment. Reaction norms have not yet been investigated in lepidopteran larvae in this context.

We used calibrated stimuli in order to investigate the adaptive significance of colour change in *B. betularia* (Stevens & Merilaita, 2009). We manipulated luminance (brightness) and colour, and evaluated the degree to which *B. betularia* caterpillars are able to respond to intermediate strength cues (i.e., discrete polyphenism vs. reaction norm). We also measured the response to heterogeneous twig colour environments. For the purpose of these experiments, 'colour' encompasses hue and chroma. Hue is defined as the direction of the colour vector, and chroma as how different a colour is from achromatic white/black (Stoddard & Prum, 2008). 'Luminance' is defined as achromatic intensity, or perceived brightness (Stoddard & Prum, 2008; Stevens *et al.*, 2014a). We modelled colour using the avian visual system which allows a more direct adaptive interpretation of larval colour change in *B. betularia*, compared to using human vision. We tested the following predictions: (1) larvae respond to both colour and luminance; (2) larvae produce intermediate

phenotypes in response to changing colour and/or luminance on a continuous scale [i.e., a reaction norm rather than a polyphenism, as suggested by Noor *et al.* (2008)]; (3) when faced with a heterogeneous background, larvae adopt an intermediate colour reflecting the relative proportion of twig colours.

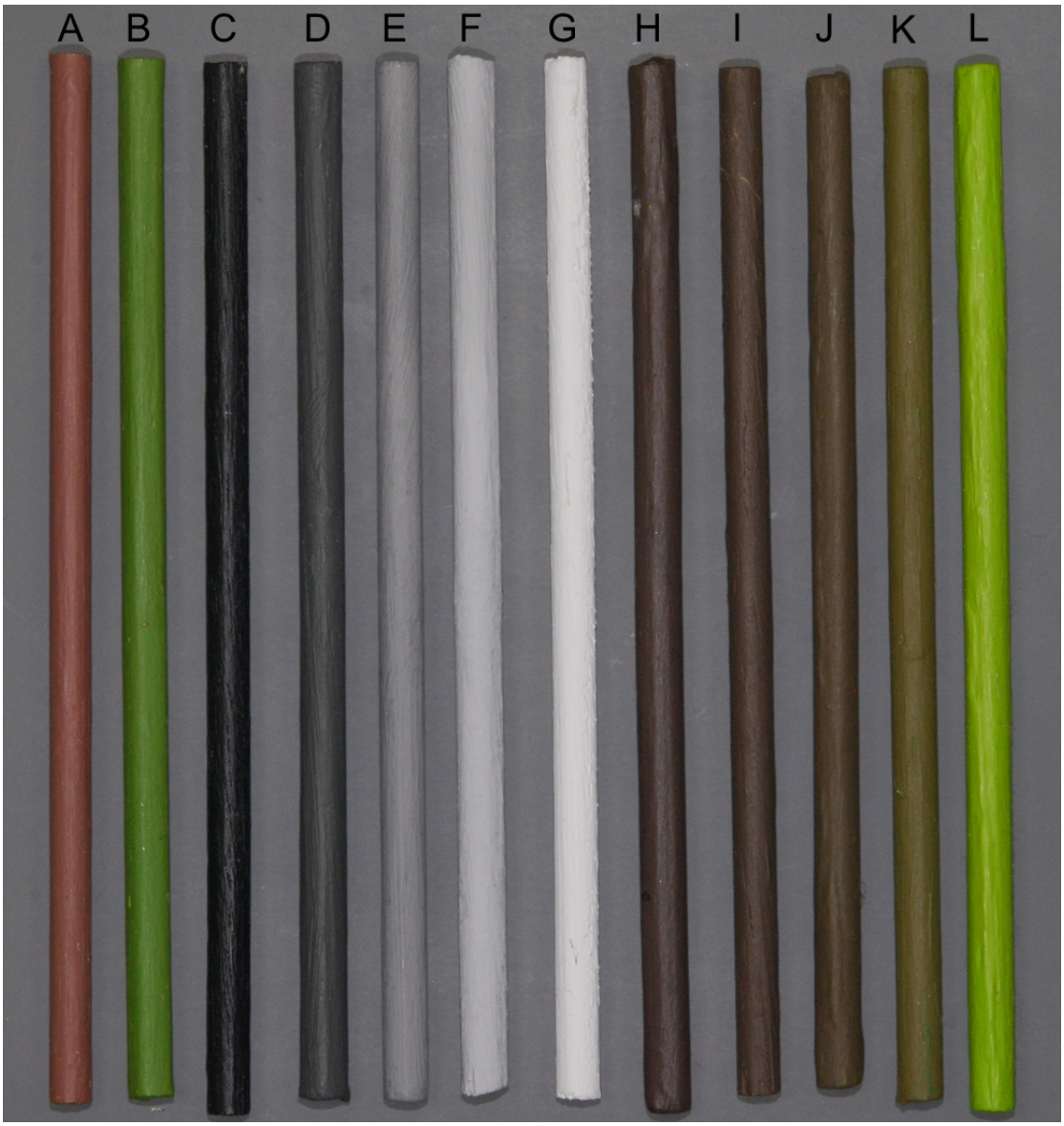


**Figure 2.2. Dowels used for luminance, colour, and heterogeneous environment experiments.** (A–L) represent IB, IG, Bl, BW1, BW2, BW3, Wh, Br, BG1, BG2, BG3, and Gr, respectively.

## MATERIALS & METHODS

### Experimental animals and rearing

All larvae for the various experiments were the $F_1$ offspring from crosses between wild-caught or captively reared adults. Larvae typically undergo five moults, resulting in six instars until pupation (Noor *et al.*, 2008). In the first instar, larvae appear countershaded, and in subsequent instars the larvae develop colours that

appear to match the twigs of the different host plants on which they may rest. The adults occur as a series of more or less discrete morphs differing in the degree of melanism: *typica*, *insularia* and *carbonaria* (Cook & Muggleton, 2003). As previous observations suggested no effect of adult morph on larval colour, or vice versa, some families used in these experiments were segregating for adult melanism alleles, whilst others were fixed for the *typica* allele (Table S2.1). For experiments requiring more larvae than one cross could provide, larvae from multiple crosses were split across treatments to minimise any family effects (Table S2.1).

Larvae were initially reared from eggs on intact goat willow (*Salix caprea*) branches with leaves until second instar. Groups of 25 caterpillars from the same family (full siblings) were then introduced into transparent plastic boxes measuring 279 x 159 x 102 mm (length x width x depth) containing an irregular lattice of twenty 12 cm-long (ten 3mm and ten 5mm diameter) painted softwood dowels. The dowels were held in place with a chicken wire mesh frame painted the same colour as the dowels (Fig. 2.2). All paints used for dowels were from the Dulux Matte range (Table S2.1). To facilitate cleaning, the base of each box was lined with a plain blue C-Fold 1-ply paper towel and larvae were fed on stripped, stalkless leaves of goat willow (*Salix caprea*), which was replenished so that the larvae had a constant supply of food. Boxes were regularly cleaned and hands and equipment were washed in dilute bleach (10%) between handling of boxes to reduce risk of disease transmission. Experiments were conducted in a Sanyo Versatile Environment Test Chamber (MLR-351), on a 12:12 hour day: night cycle at 24$^{o}$c in the day with light intensity set at 15000 lx, and 18$^{o}$c at night. Boxes were arranged two on each shelf, 20 cm apart, leaving a 60 cm height space with a shelf between boxes.

**Colour and luminance quantification**

***Spectrophotometric analysis***

Reflectance measurements of larvae and dowels were taken using an Ocean optics USB2000 spectrophotometer, with specimens illuminated at 45º to normal by a DH1000 balanced halogen deuterium light source. The measuring spot diameter was 3 mm, with spectra recorded at 0.34 nm intervals from 300 to 700 nm and measured relative to a WS-1 reflectance standard (for raw spectra, see Figs. S2.5, S2.6). Spectrophotometry data were visualised using Overture (v.1.0.1). Reflectance spectra were reduced to 1 nm intervals within the 300-700 nm range using customised code (provided by I. Cuthill).

Firstly, to determine differences in 'colour' between larvae and the dowels from the colour experiment, the predicted photon catches of cone types long wavelength (LW), medium wavelength (MW), short wavelength (SW), ultraviolet (UV) and double dorsal (DD) of a blue tit, *Cyanistes caerulys,* were modelled for each spectrum in tetrahedral colour space following the Vorobyev-Osorio model (Vorobyev & Osorio, 1998), using a program written in Matlab (Cuthill, 2006). We modelled larval colour from the perspective of an avian predator rather than the human eye, because if colour change in *B. betularia* is an adaptation to avoid predation, it would likely be driven by the natural predator. We used the blue tit to represent the avian visual system because the spectral sensitivities for this species have been measured and made available, and blue tits are known to eat geometrid larvae (Arnold *et al.*, 2010). Cone stimulation values were converted to Cartesian coordinates and plotted in a tetrahedral space using a Matlab program (Stoddard & Prum, 2008), such that each cone is represented by an axis. This colour space is useful because if a colour stimulates only one cone type, then its coordinates lie at the appropriate tip of the tetrahedron, and when all four cone types are equally stimulated the point lies at the origin. To provide a simpler measure of colour, we calculated greenness as the ratios between the cone catch values of the medium wavelength and long wavelength photoreceptors (MW/(MW+LW), which represent opponent mechanisms, following Arenas and Stevens (2017). For the achromatic dowel experiment we created a stimulus that increased in luminance in the absence of 'colour' (black to white), therefore we did not model response to colour, only luminance. We analysed only the blue tit double dorsal cone catch, as these cones mediate luminance vision (Campenhausen & Kirschfeld, 1998; Osorio & Vorobyev, 2005).

We modelled the ease with which an avian predator might discriminate between dowels and larvae using just noticeable differences (JND; see Vorobyev and Osorio, 1998 for equations). For chromatic contrasts, we used spectral sensitivities of the blue tit using relative cone ratios of SW=0.7111; MW=0.9926; LW=1.0 and UV=0.3704 (Hart *et al.*, 2000), with a Weber fraction of 0.05 and idealized irradiance (D65). To model luminance JNDs, we used blue tit double dorsal (DD) cones. JND <1.00 indicate that two stimuli are indiscriminable; stimuli differing by 1-3 JND units are only discriminable under good viewing conditions; and stimuli

showing values above this should be distinguishable with increasing ease (Stevens *et al.*, 2015).

***Photographic analysis***

Colour/luminance analysis on larvae from the heterogeneous dowel experiments was performed using calibrated photographs, as the spectrophotometer was not available when these experiments were conducted. Photographs of individual larvae were normalised to a standardised grey background (18%) and linearised to 32-bit files using the Image Calibration and Analysis Toolbox (Troscianko & Stevens, 2015) in ImageJ (v.1.49p). RGB values were extracted from processed images using ImageJ from an average of six dorso-lateral measurements per larva: one from each side of the 3rd thoracic, and 2$^{nd}$ and 6$^{th}$ abdominal segments. An average measure of percentage greenness was then calculated across the six measurements using G/(R+G+B)*100 from RGB ratios. Although objective, these measurements were not modelled using an avian visual system.

**Experimental treatments**

A total of four experiments were conducted to test three main hypotheses concerning the nature of the environmental cue and the phenotypic response (Table 1).

**Table 1.** Summary of experiments and hypotheses

| Experiment | Twig environment | Hypothesis |
|---|---|---|
| i | contrasting colour | 1a. larvae respond to differences in twig colour |
| ii | luminance gradient | 1b. larvae respond to differences in twig luminance<br>2a. larvae can produce intermediate responses to twig luminance |
| iii | Colour and luminance gradient | 2b. larvae can produce intermediate responses to twig colour and luminance |
| iv | Heterogeneous environment | 3. larvae generalise across twig colours |

***(i) Colour treatments***

Dowels were painted either isoluminant green or isoluminant brown (Fig. 2.2: IG, IB) to create two treatments that differed in overall colour (colour JND: 21.2) and greenness (Two sample t-test, $t_{9.99}$= -16.86, $P$<0.0001), but not luminance (luminance JND: 1.8, Two sample t-test, $t_{5.86}$ = 0.37603, $P$=0.7201). Five replicate boxes were used for each treatment (Table S2.1). Larvae were reared on a 12:12 hour day: night cycle, at 24°C in the day and 18°C at night. Once larvae had reached final instar, six reflectance measurements per larva were taken with a spectrophotometer,

three from each lateral surface, on the 3rd thoracic, and 2nd and 6th abdominal segments. These segments were chosen to obtain repeated measurements of the main body colour, excluding any prominent markings.

***(ii) Luminance gradient***

Five luminance treatments were created using painted dowels (Fig. 2.2: B1, BW1, BW2, BW3, Wh) increasing in luminance (ANOVA, $F_4$=8415, $P$<0.0001) from near-black to white (Fig. S2.1A, Table S2.1), and approximately equal in colour. Paint was matched to the grey standards from a Gretag Macbeth colour chart using a Gretag Macbeth colour scanner at a UK hardware store (B&Q). Three replicate boxes per treatment were used (Table S2.1). Larvae were reared on a 15:9 hour day: night cycle at 21°C in the day and 19°C at night. Once larvae had reached final instar, four reflectance measurements were taken with a spectrophotometer from the dorsal surface of each caterpillar, on the 3rd thoracic segment, and the 2nd, 4th and 6th abdominal segments.

***(iii) Colour and luminance gradient***

We mixed brown (Br) and green (Gr) paint in three different ratios to give a total of five treatments that ranged from brown to green (Table S2.1, Fig. 2.2: Br, BG1, BG2, BG3, Gr). These treatments differed in greenness (Fig. S2.1D; ANOVA, $F_4$= 1378, $P$<0.0001) and luminance (Fig. S2.1C; ANOVA, $F_4$=82.68, P<0.0001), although greenness of BG1, BG2 and BG3 was less than expected based on the proportion of Gr paint in the mixture. Three replicate boxes per treatment were used. Larvae were reared on a 12:12 hour day: night cycle, at 24°C in the day and 18°C at night. Once larvae had reached final instar, six reflectance measurements per larva were taken as for colour treatments.

***(iv) Heterogeneous dowel environment***

Five treatments were created using only two colours of dowel, brown and green (Fig. 2.2: Br, Gr), but in different ratios: 100% brown, 70 brown: 30 green, 50 brown: 50: green, 30 brown: 70 green, and 100% green (Table S2.1). Larvae were reared on a 15:9 hour day: night cycle at 21°C in the day and 19°C at night. Once the final instar was reached, the dorsal surface of each larva was photographed on a standard grey card background using a Nikon D80 digital camera, 60 mm macro lens with the following settings: 1/60s (shutter speed), 16 (F), 400 (ISO), cloudy (white balance), 2x Nikon Speedlight SB-400 External Flash.

**Statistical analyses**

All statistical analyses were performed using R version 3.1.0 (R Core Team, 2014) . Responses to contrasting colour cues were compared using a linear mixed model in the lme4 package in R (Bates *et al.*, 2015), with replicate nested within treatment. Luminance gradient and greenness gradient response means for each of the five treatment levels were compared using a one-way ANOVA. Polynomial models from orders 1-4 were fitted to the luminance and greenness correlations to explore the relationship between environmental gradient and larval response. All polynomial models are presented as fitted (see Fig. S2.2). $R^2$ value, visual fit, and examination of plotted residuals were used to determine the best model for each correlation. ANOVA was performed to look for significant differences in fit between models. The 'greenness' response of larvae reared under different degrees of dowel-colour heterogeneity was analysed by comparing means using a one-way ANOVA. Homogeneity of variance between treatment medians was explored using Levene's test.

**RESULTS**

**General response and observations**

Colour change in *B. betularia* larvae took approximately 14-21 days to complete, depending on the individual. Noticeable change began to occur around 7 days after larvae were placed on stimuli and occurred within each moult, as well as between moults. Mortality occurred at a rate of 5-10% in each treatment box, usually during the first half of larval development.

**Response to colour (isoluminant dowels)**

Larvae presented with the isoluminant green (IG) treatment were significantly greener than those in the isoluminant brown (IB) treatment (Fig. 2.3A; $F_{11,\ 125}$= 33.69, $P$<0.0001). In colour space, the larvae resembled their own treatment colour more closely than the alternative treatment colour (Fig. 2.3B), and green and brown larvae were discriminable to a bird (colour JND: 11.3). The average response was consistent across replicates within treatments (Fig. 2.3C), but the discrepancy between larval and dowel greenness was greater for larvae reared on green dowels (colour JND: 9.9), than those reared on brown dowels (colour JND: 8.3).

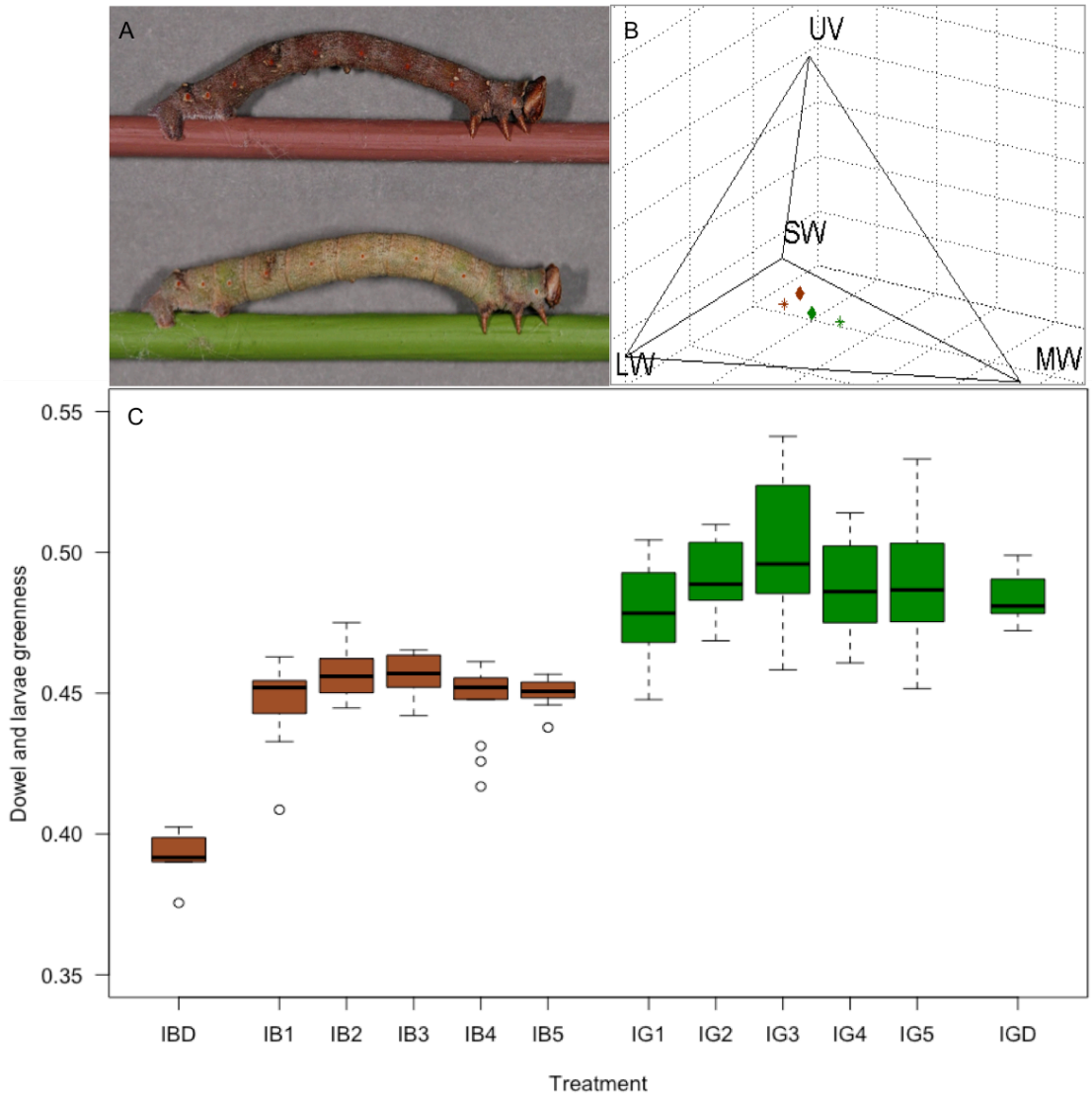


**Figure 2.3. The response of *B. betularia* larvae to a difference in dowel colour.** (A) Representative final instar *B. betularia* larvae from each isoluminant treatment resting on their corresponding dowel. (B) The average position of final instar *B. betularia* larvae and their corresponding dowels within the ultraviolet-sensitive (UVS) avian tetrahedral colour space when viewed by a blue tit, *Cyanistes caeruleus*, under bright daylight conditions. Asterisks represent dowels, rhombuses represent larvae from brown and green treatments, respectively. The plot illustrates the stimulation of the short (S), medium (M), long (L), and UV (U/V) wavelength-sensitive photoreceptors and is shown from the MW–LW plane. (C) Greenness as perceived by a blue tit under bright daylight conditions of final instar *B. betularia* larvae reared under isoluminant dowel treatments, where IB= isoluminant brown larvae and IG= isoluminant green larvae. The numbers following the letters indicate replicate boxes within each treatment. IBD= isoluminant brown dowel and IGD= isoluminant green dowel. Boxes represent median (midline) ± interquartile range (IQR), whiskers represent IQR ± (1.5 x IQR), open circles= outliers. Photo credit: Arjèn Van't Hof.

**Response to luminance gradient**

Larvae responded to dowel luminance ($F_4$= 148.2, $P$<0.0001), ranging from very pale on white dowels to very dark on black dowels, with intermediate degrees of luminance on grey dowels (Fig. 2.4A). The relationship between larvae and dowel luminance was significantly cubic ($F_{3,\ 261}$=156.3, $P$<0.0001, $R^2$= 0.64): relatively steep at the extremes and shallow at intermediate luminance (Fig. 2.4B). This shape was due to smaller luminance differences between larvae from intermediate treatments (BW1 vs. BW2: luminance JND: 1.2; BW2 vs. BW3 luminance JND 3.4). The differences between larvae from the two extremes of the gradient (black and white and intermediate were larger (Bl vs. BW1, luminance JND: 17.4; and Wh vs. BW3, luminance JND: 13.2).

When comparing larvae to their corresponding dowels, larvae from higher luminance treatments (BW2, BW3 and Wh) were most different from their dowels (luminance JNDs: 35.9, 43.4 and 35.8, respectively). Black (Bl) and dark grey (BW1) treatments showed comparatively lower JNDs between larvae and dowels (luminance JNDs: 28.9 and 20.0, respectively).

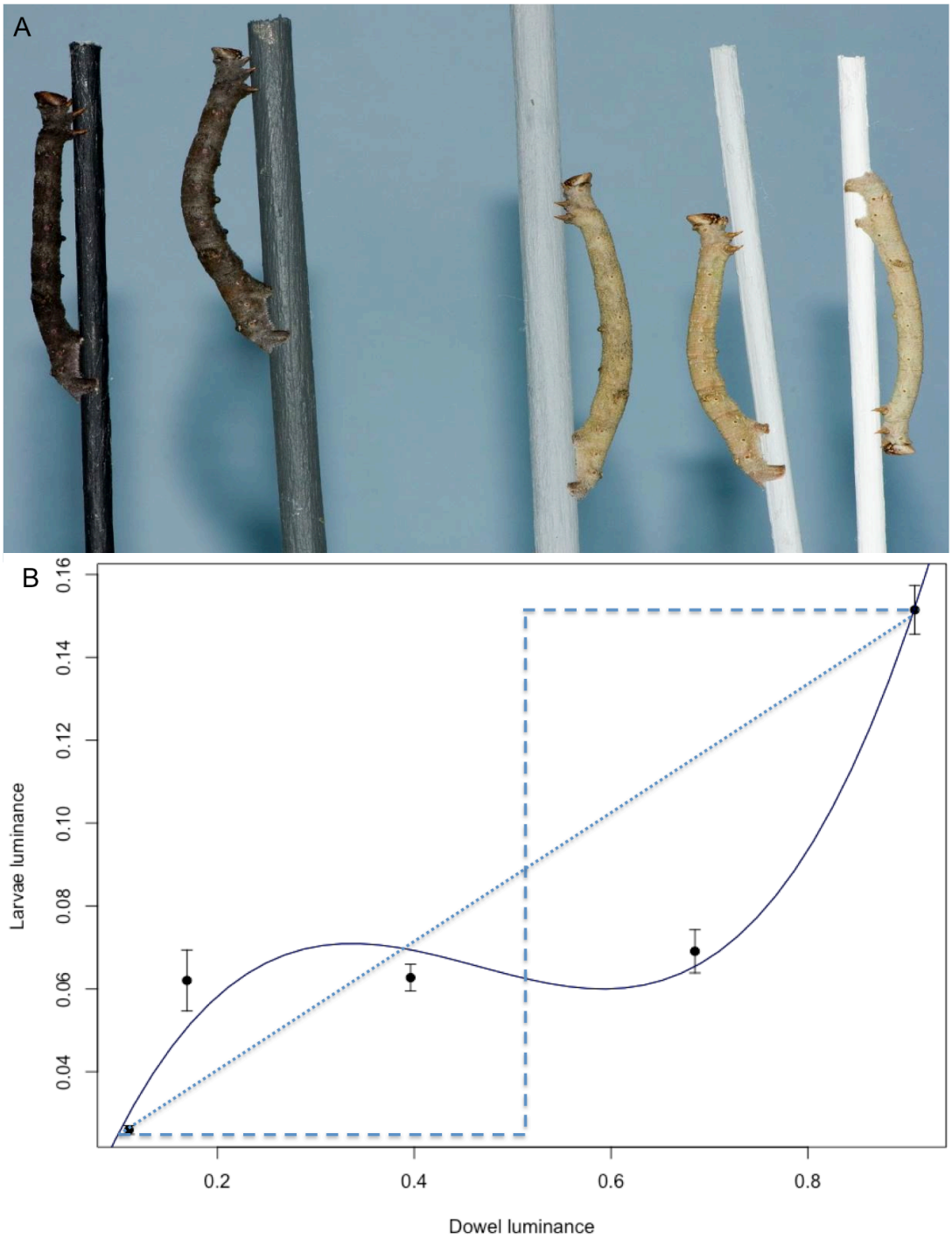


**Figure 2.4. The response of *B. betularia* larvae to a gradient in dowel luminance.** (A) Representative final instar *B. betularia* larvae from each luminance treatment resting on their corresponding dowel. Dowel treatments shown from left to right: Black (Bl), Dark grey (BW1), Mid grey (BW2), Light grey (BW3), White (Wh). (B) Average luminance of final instar *B. betularia* larvae reared under the five luminance treatments, as perceived by a blue tit (*Cyanistes caeruleus*) under bright daylight conditions. Solid line is the fitted cubic polynomial; dotted and dashed lines, provided for comparison, represent the linear (idealised continuous reaction norm) and stepped (two-state polyphenism) responses, respectively. Photo credit: Arjèn Van't Hof.

**Response to colour and luminance gradient**

*B. betularia* larvae adjusted both greenness ($F_4$=120.6, $P$<0.0001) and luminance ($F_4$=82.68, $P$<0.0001) in response to dowel stimuli (Fig. 2.5A), showing a significant positive quadratic correlation between larvae and dowel greenness (Fig. 2.5B; $F_{2, 277}$= 225.6, $R^2$=0.62, $P$<0.0001). Two of the intermediate brown-green treatments (BG1 and BG2) were very close in greenness (Fig. 2.5B), and discrimination between them was low (colour JND: 4.61, luminance JND: 2.9). Larvae from these treatments followed this pattern closely, with identical greenness of 0.45 (Fig. 2.5B) and low discrimination values (colour JND: 2.4, luminance JND: 2.9). The colour discrepancy between larvae and dowels from the brown treatment (Br) was smaller (colour JND: 5.8) than for the green (Gr) treatment (colour JND: 14.1).

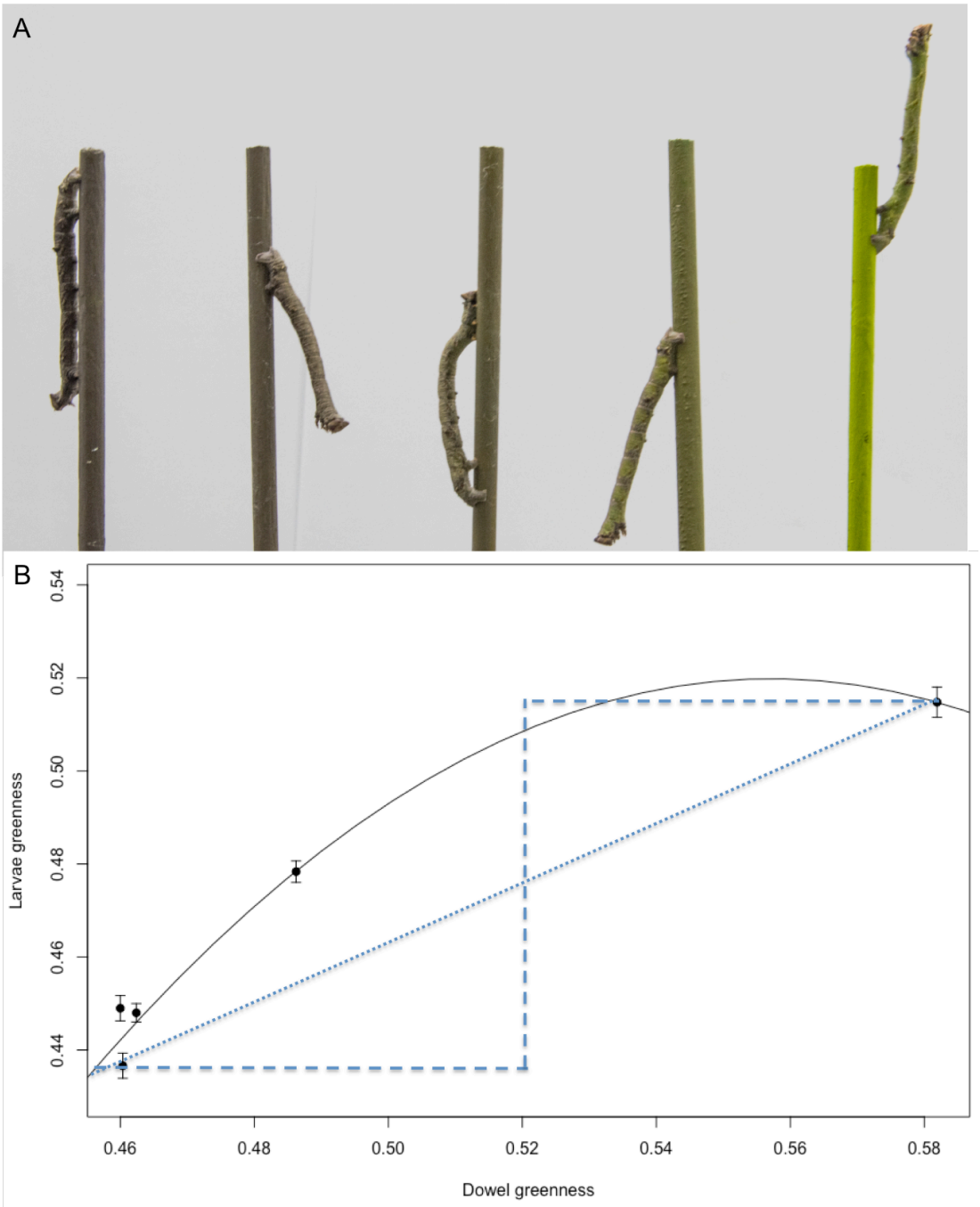


**Figure 2.5. The response of *B. betularia* larvae to a gradient in dowel colour and luminance.** (A) Photograph of final instar *B. betularia* larvae from each colour treatment resting on their corresponding dowel. Dowel treatments shown from left to right: Brown (Br), More brown (BG1), Brown-green (BG2), More green (BG3), Green (Gr). (B) Average greenness of dowels vs. *B. betularia* larvae exposed to dowels from each of the five treatment groups, as perceived by a blue tit (*Cyanistes caeruleus*) under bright daylight conditions. Solid line is the fitted quadratic polynomial; dotted and dashed lines provided for comparison represent the linear (idealised continuous reaction norm) and stepped (two-state polyphenism) responses, respectively. Photo credit: Lukasz Lukomski.

### Response to heterogeneous colour environments

As the proportion of green dowels relative to brown dowels in each enclosure increased, the average greenness of *B. betularia* larvae in each enclosure also increased (Fig. 2.6; $F_4$=16.2, $P$<0.0001). Between-individual variance in larval

greenness was significantly higher in the three heterogeneous than in the two homogeneous colour environments (Levene's test, $F_4$=16.558, $P$<0.0001). This result still held when the most variable treatment was removed (Levene's test, $F_3$=8.3093, $P$<0.0001). The apparent change in the average level of greenness in mixed treatments (Fig. 2.6) arose predominantly from changes to the ratio of 'green': 'brown' individuals, which was roughly in line with the dowel ratios, rather than every larva taking on an intermediate colour (Fig. S3).

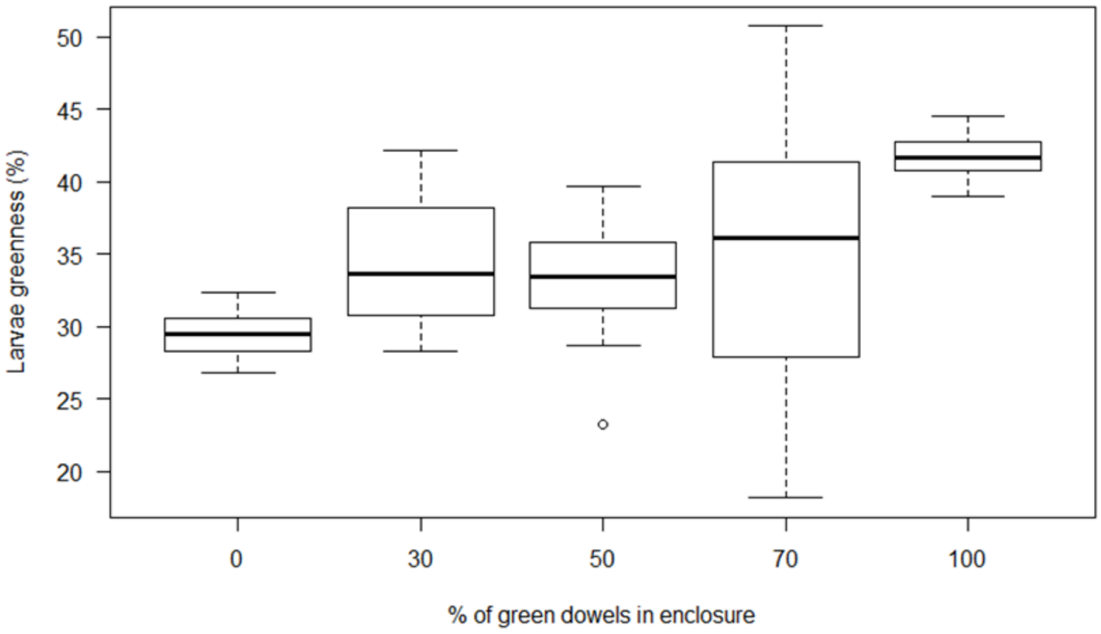


**Figure 2.6. The response of *B. betularia* larvae to different ratios of green and brown dowels.** Percentage of green dowels in each treatment *vs*. the percentage of greenness of *B. betularia* larva as calculated by RGB analysis. Boxes represent median (midline) ± interquartile range (IQR), whiskers represent IQR ± (1.5 x IQR), open circles= outliers.

## DISCUSSION

*Biston betularia* caterpillars changed colour to match the twigs upon which they rested, across all four experiments. Our results support the prediction that larvae would be able to respond to both colour and luminance (hypotheses 1a and 1b in Table 1). Larvae reared on green and brown dowels changed colour to match those dowels, and larvae reared on black and white dowels changed their luminance. Many other species can adjust luminance to enhance their camouflage from predators, such as flounders, sand fleas, and toads (Fairchild & Howell, 2004; Stevens *et al.*, 2015; Polo-Cavia *et al.*, 2016). Frogs, gobies and cephalopods can rapidly adjust colour in response to visual backgrounds using chromatophores (Mathger & Hanlon, 2007; Hanlon *et al.*, 2009; Stevens *et al.*, 2014a; Kang *et al.*, 2016). To our knowledge, our results are the first to show that lepidopteran larvae respond to both colour and

luminance, and are likely to use dermal pigments as opposed to chromatophores to change their appearance.

We also found that when *B. betularia* larvae were presented with colour and luminance gradients, the larvae produced intermediate phenotypes, on a continuous scale, to approximately match each background. This supports our second prediction (hypotheses 2a and 2b, Table 1). Intermediate phenotypes have been reported in amphibians, fish, and a number of benthic invertebrates, but the cues and mechanisms controlling these phenotypes have not been rigorously explored (Lin *et al.*, 2009; Skold *et al.*, 2013; de Bruyn & Gosselin, 2014; Kang *et al.*, 2016). Contrary to our third hypothesis (Table 1), larvae did show an increase in *average* greenness across the treatments with a heterogeneous background. However, this was largely due to an increasing proportion of green individuals compared with brown individuals, rather than every larva becoming greener. Our four experiments provide the first conclusive evidence of intermediate colour change in lepidopteran larvae in response to visually graded or heterogeneous cues. Our results extend our understanding of the type of visual cues that *B. betularia* larvae use for colour change, and the range of colours they can produce.

Visual control of colour change is well known in animals that exhibit rapid colour change, such as flatfish (Kelman *et al.*, 2006) and cephalopods (Mathger & Hanlon, 2007; Ramirez & Oakley, 2015), where chromatophores, under direct control from the visual nerve system, are responsible for the rapid colour change (Messenger, 2001; Kingston *et al.*, 2015). Other species show comparatively slower responses to background manipulation. For example, shore crabs respond predominantly to luminance cues over colour to match their background (Stevens *et al.*, 2014b), and sand fleas are able to match changes in luminance and colour to avoid predation (Stevens *et al.*, 2015). In Lepidoptera, early experimental evidence indicated that colour change is associated with larvae and pupae sensing their visual environment (Poulton, 1890). Since this pioneering work, the evidence collected in support of visually induced colour change in Lepidoptera has been limited and inconclusive: the experiments in *B. betularia* larvae (Noor *et al.*, 2008), and two species of hawkmoth larvae, *Smerinthus ocellata* and *Laothoe populi* (Grayson & Edmunds, 1989) did not measure colour objectively from the perspective of an ecologically relevant predator, and other potential cues were not controlled for.

In showing that background colour induces the phenotypic change in *B. betularia*, our results are in accordance with some of the conclusions drawn by Noor *et al.* (2008). By keeping dietary and tactile cues constant, we also found that *B. betularia* larvae use visual cues to change colour. However, our results differ from Noor et al. (2008) in that we have found that the response is a continuous reaction norm, not a polyphenism of only two phenotypes. This may be because the experiments by Noor *et al.* (2008) did not provide a spectrum of background colours, or because the responses of the larvae in Noor et al. (2008) were measured subjectively by assigning individual caterpillars as ‘‘best fits’’ to one of four colour categories. This necessarily reduces any variability to four levels. We objectively measured the colour of both the stimuli and the larvae from the perspective of avian predators. This information provides us with a better understanding of how the highly polyphagous larvae of *B. betularia* might avoid predation in a changing environment. The range of colour phenotypes that the larvae are able to produce could enable crypsis on a wide range of host plants, reducing costs of lost foraging opportunity, and explaining the higher probability of polyphagy by masquerading species (Ruxton *et al.*, 2004). The background matching ability of *B. betularia* larvae is also likely to allow them to adapt to the blackening of trees and shrubs caused by atmospheric coal pollution. There is no direct evidence for this in *B. betularia,* as its larvae are very difficult to collect from the wild and occur at low densities. However, the twig-mimicking caterpillars of another geometrid, *Odontopera bidentata*, which are normally pale to medium brown, or with green (‘lichen’) patches, were uniformly black in the heavily polluted inner-city areas of 1970’s Manchester (Bishop & Cook, 1980).

The continuous relationship we observed between dowel colour and larval colour is non-linear, whereas the standard reaction norm is generally depicted as a linear relationship (Oomen & Hutchings, 2015). Non-linear reaction norms are common in nature; for example, in response to temperature:life history in butterflies (Brakefield *et al.*, 1998), pigmentation in fruit flies (Rocha *et al.*, 2009), and morphology in sticklebacks (Ramler *et al.*, 2014). The reason that we observed a non-linear relationship may be because colour change is costly (Polo-Cavia & Gomez-Mestre, 2017). However, the cost of colour change and the trade-off between these and foraging costs has yet to be explored in this species. An alternative explanation is

that our stimuli did not surpass the thresholds needed to elicit the cascade from vision to colour (Burtt, 1951).

Vision in lepidopteran larvae has been much less studied than the compound eye of the adult stage (Briscoe & Bernard, 2005; Xu *et al.*, 2013; Liu *et al.*, 2017), but it is assumed that the simple ring of eyes or ocelli provides relatively poor vision (Ichikawa, 1990; Lin *et al.*, 2002). Our results show that *B. betularia* larvae can perceive differences in brightness and colour, and support the idea that visually induced plastic colour change in arthropods is mediated through the eyes. There is also growing evidence that camouflage may be partly guided by light-detecting opsin proteins outside the eye (Stevens, 2016). Further exploration of the visual processes and pathways that determine the sensitivity and range of colour change is important for understanding camouflage, and has been highlighted as a key area for future work (Duarte *et al.*, 2017).

In our experiments, there was variation in the degree of colour and luminance matching of the larvae to the dowels. For example, larvae were able to increase luminance as dowel luminance increased, but were always less bright than their corresponding dowels and in some cases would be detectable by birds. Larvae were also not able to closely match the green (Gr) dowel, and again would be detectable by birds. However, we know that resembling twigs is sufficient for masquerade to reduce predation risk, and a lack of perfect colour/luminance match is less detrimental for masqueraders than for cryptic prey (Skelhorn *et al.*, 2010c). This lack of perfect resemblance could be due to physiological constraints, as the white dowels were highly luminant and the green dowels had a very high greenness score. The colours we used were chosen to test the range of colours that *B. betularia* larvae were able to match, rather than closely resembling the colour/ luminance of twigs that individuals are exposed to in nature.

The physiological mechanism behind the colour change in *B. betularia* is unknown, though preliminary investigations have revealed that the external colour is achieved by varying pigmentation in three primary layers of epidermal tissue (Fig. S4). Cuticular pigments are responsible for colour patterns and have been described in other lepidopteran larvae (Goodwin, 1953; Dahlman, 1969). It is assumed that sequestering bright white or green pigments through a plant diet may be difficult, but yellow and white colouration is achieved with ommochrome pigments in the crab

spider, *Misumena vatia* (Insausti & Casas, 2008). Material properties may also affect luminance, as different materials reflect different amounts of light, which may be the reason for *B. betularia* larvae achieving lower luminance than dowels. Another explanation for the larvae not achieving a perfect match to their backgrounds is similar to the ideas on imperfect mimicry (Greene & McDiarmid, 1981; Pekar & Jarab, 2011). Masquerade alone enables larvae like *B. betularia* to avoid being eaten by birds (Skelhorn *et al.*, 2010b); therefore, if there is weak selection against imperfect mimics, then imperfect colour and pattern may not entirely negate the deceptive effect. *Kallima* butterflies masquerade as dead leaves, closely matching the shape, texture, and colour of the leaf (Suzuki *et al.*, 2014). It is not currently known whether the shape (and posture), or colour is more important in remaining inconspicuous to predators, but it is thought that relaxed selection on close colour mimicry may occur because cognitive processes of predators (learned discrimination) are more important than sensory processing for visual detection of prey (Stoddard, 2012). Relaxed selection has been suggested as a precursor to phenotypic plasticity (Hunt *et al.*, 2011), and relaxed colour selection in *B. betularia* could have contributed to colour plasticity in the larvae. However, more research is needed on this topic to understand the origins of colour plasticity in antipredator defences.

## CONCLUSIONS

We show that larvae of the peppered moth use visual cues to closely match the colour and luminance of their background and that this is a continuous response, or reaction norm. The adult and larval stages of *B. betularia* show alternative evolutionary routes to crypsis, with colour polymorphism under genetic control in the adult moths and reaction norm in the larvae. Both routes achieve protection against avian predation, and it is likely that these contrasting evolutionary strategies have been influenced by differences in life history traits, such as dispersal, reproduction, and feeding behaviour between adults and larvae, as well as physiology. Our results show a novel response in a species belonging to a group of animals whose camouflage potential has been poorly studied in comparison to other taxonomic groups.

# Chapter 3

## Extraocular photoreception: colour response of blindfolded caterpillars

**ABSTRACT**

Many prey animals can increase concealment from predators by improving their match to the colour and/or pattern of their surroundings through plastic colour change. Previous studies have suggested that visual cues initiate the colour change cascade in some animals, but in many cases this evidence is not conclusive. There is also growing evidence for photoreception occurring outside of the eye, but the functions of these photoreceptors in the context of colour change are rarely described. Here, we show that peppered moth (*B. betularia*) larvae are able to respond to and match differences in chroma and luminance of the twigs they rest on, without the use of their eyes. It is likely that dermal photoreception enables *B. betularia* larvae to achieve uniform colour change whilst resting at an angle from the twigs that they mimic. This is the first example of extraocular photoreceptors guiding colour change in lepidopteran larvae, and it is possible that this phenomenon is not restricted to this species.

**INTRODUCTION**

Colour change for the purpose of background matching is arguably the most common form of camouflage, and is widespread in the animal kingdom (Stevens & Merilaita, 2009). Some animals have the ability to perceive their visual background and use this information to adjust their body colour or luminance to match it, remaining inconspicuous to predators (Stevens *et al.*, 2014a; Stevens *et al.*, 2014b; Kang *et al.*, 2016). Responding to visual cues for background matching could be advantageous over dietary or seasonal cues (Greene, 1989; Yamasaki *et al.*, 2009; Mills *et al.*, 2013), as an animal can use direct colour information from the environment, whereas seasons or host plants do not always correspond with background colour, causing environment-phenotype mismatches (Cook *et al.*, 2012; Mills *et al.*, 2013).

It is assumed that if animals are able to match the colours in their visual environments, they must have the visual capability to sense and discriminate between differences in hue and brightness (Sumner, 1911; Stevens *et al.*, 2015; Kang *et al.*, 2016). Most animals, including many invertebrates have complex, image-forming eyes. These are comprised of thousands of photoreceptor cells organised into a retina, which is capable of colour discrimination using opsin proteins that are

sensitive to different wavelengths in the visible spectrum (Shichida & Matsuyama, 2009). Cephalopods, reptiles, amphibians, and fish have the ability to change colours and patterns rapidly, in as little as milliseconds, to match their visual backgrounds (Mathger *et al.*, 2003; Mathger & Hanlon, 2007; Kindermann & Hero, 2016).

In the last decade, substantial research effort has been invested into understanding the physiology of this fast colour change, including visual processing in these animals (Duarte *et al.*, 2017). Rock gobies are able to change colour to match manipulated red and blue backgrounds (Stevens *et al.*, 2014a), and their eyes contain three cone pigments that can discriminate between short (blue), medium and long (red) wavelengths (Utne-Palm & Bowmaker, 2006). Cephalopods such as octopus and cuttlefish are colour-blind and express only one visual pigment in the retina (Brown & Brown, 1958; Messenger, 1977; Mathger *et al.*, 2006), but can still produce a large repertoire of colours for background matching, disruptive colouration, and social signalling (Barbato *et al.*, 2007; Mathger & Hanlon, 2007; Hanlon *et al.*, 2009; Buresch *et al.*, 2011). Dynamic colour change in cephalopods is attained by the contraction of pigmented organelles in the dermal tissue called chromatophores (Cloney & Brocco, 1983; Messenger, 2001). Recent studies have discovered opsin gene expression in chromatophores, providing evidence for visual phototransduction, and therefore dermal light sensing in squid, octopus and cuttlefish (Kingston *et al.*, 2015; Ramirez & Oakley, 2015).

Light sensing outside the eye is not uncommon, having been reported in several animal groups, including reptiles, amphibians, arthropods, and molluscs (Mrosovsky & Tress, 1966; Pankey *et al.*, 2010; Fulgione *et al.*, 2014). Extraocular photoreceptors (EOPs) in some animals are organised into organs similar to eyes, such as the pineal eye in amphibians and reptiles, or H-B eyelets and Bolwig's organ in *Drosophila*, responsible for circadian rhythm (Hofbauer & Buchner, 1989; Tosini, 1997; Veleri *et al.*, 2007). Alternatively, 'dermal light sense' coined by Millot (1968), refers to a widespread photic sense not mediated by the eye. Little progress has been made in understanding dermal photoreception since Millot's (1968) work. In many cases, behaviours that may be associated with EOPs have yet to be linked to sensory cells or biochemical pathways that mediate them (Ramirez *et al.*, 2011). Many of the behaviours associated with EOPs are phototaxes, the directional movement of an animal towards or away from light (Jekely, 2009). Two eyeless bivalve species, *Lasea rubra* and *Mya spp.*, retract their siphons in response to

changes in illumination and have photoreceptors located in the foot and siphon (Light, 1930; Morton, 1960). The pond snail, *Lymnaea stagnalis*, uses dermal photoreceptors located on the foot for phototaxis and shadow response (Chono *et al.*, 2002). Examples of EOP mediated behaviour have been reported in arthropods. For example; adult Swallowtail butterflies, *Papilio xuthus*, have pairs of photoreceptors localised to the genitalia, which are used to control copulation in males and assist in oviposition in females (Arikawa & Miyako-Shimazaki, 1996; Arikawa *et al.*, 1997). Many of the described behaviours mediated by EOPs: shadow response, light avoidance, circadian rhythm setting, and substrate preference, do not require colour or image-forming vision (Ramirez *et al.*, 2011). However, there may be some EOP networks that have not yet been described that may aid in behaviours that utilise colour vision, such as camouflage.

Slow colour change has been studied far less extensively than the rapid colour change achieved through chromatophores (Stevens, 2016). However, there is some evidence describing comparatively slower matching for visual backgrounds; for example, the chameleon prawn, *Hippolyte varians*, changes colour relatively slowly (approximately one week), to match different visual backgrounds, producing a range of colours including red, brown, green and yellow (Keeble & Gamble, 1899). The colour change in *Thomisus onustus* crab spiders is also slow, taking 5-25 days depending on the colour, and was found to be in response to visual cues (Insausti *et al.*, 2012). The colour patterns in crab spiders are produced by the dermal distribution of ommochrome pigments synthesised from tryptophan (Llandres *et al.*, 2013; Shamim *et al.*, 2014). Slow colour change has been reported in lepidopteran larvae; for example, in two hawkmoth larvae species, *Laothoe populi* and *Smerinthus ocellatus*, colour change occurred in response to different wavelengths of light, but unfortunately, other cues (e.g. diet) were not controlled for (Grayson & Edmunds, 1989).

Caterpillars of the peppered moth, *Biston betularia*, change colour to match their visual background (Noor *et al.*, 2008), and are able to respond to luminance and hue independently (chapter 2). After eliminating other possible cues (diet and texture), we assumed that *B. betularia* larvae use visual information to match their surrounding environment (Eacock *et al.*, 2017). As in most arthropod larvae, *B. betularia* possess simple eyes, known as ocelli or stemmata on each side of their head (Gilbert, 1994). In contrast to compound eye vision in adult arthropods, which

has been extensively studied (Sison-Mangus *et al.*, 2006; Briscoe, 2008; Bybee *et al.*, 2011), information about larval vision through simple eyes is very limited. However, it is generally assumed that in comparison to the ommatidia, ocelli provide larvae with very basic vision (Lin *et al.*, 2002). This observation is unusual, regarding the accuracy and continuous nature of the observed colour change response in *B. betularia* (Chapter 2). We therefore designed experiments to explore the possibility that colour change in *B. betularia* larvae is guided by additional photoreceptors outside of the ocelli, and the capacity of such EOPs to detect colour separately from luminance. In these experiments, ocular vision was obscured with acrylic paint, and the colour response of larvae to dowel colour was measured.

## METHODS

### Animals and treatment arenas

To minimise any family effects on colour change responses, a split family design was used in all treatments (Table 3.1). *Biston betularia* were reared from eggs and fed a constant supply of goat willow (*Salix caprea*). At second instar, 25 variably colored larvae were transferred to each treatment arena (Fig. S3.1). Treatment arenas comprised of transparent plastic boxes measuring 279 x 159 x 102 mm (length x width x depth) lined with plain blue C-fold 1-ply paper towel, each box contained 20 x 12 cm-long wooden dowels (10 x 5 mm diameter and 10 x 3 mm diameter) held in position by a chicken-wire frame painted to match the colors of the dowels used for each experiment (Table 3.1). Larvae were fed a constant supply of *Salix caprea* leaves stripped from the branches and stem, and boxes were washed with 10% bleach every three days to reduce infection risk to larvae. Treatment boxes were kept 20 cm apart in a Sanyo Versatile Environment Test Chamber (model MLR-351), with a 12:12 hour day: night cycle, at 24$^{o}$C in the day with luminescence set at 15,000 lux, and 18$^{o}$C at night for the duration of the experiment, until larvae reached final instar.

**Table 3.1.** Summary of the design of the blindfolding experiments

| Year | Treatment | Dowel paint | Family ID | Number of replicates (boxes) | Number of individuals measured per replicate | Total *n* (control/painted) |
|---|---|---|---|---|---|---|
| 2013 | Blindfolded extreme luminance green | Indian ivy 3 | #199 | 3 total- 1 x control (GC1), 2x blindfolded (GP1, GP2) | GC1: 10, GP1: 13, GP2: 12 | 10/25 |
| | Blindfolded extreme luminance brown | Espresso shot | | 3 total- 1 x control (BC1), 2x blindfolded (BP1, BP2) | BC1: 15, BP1: 11, BP2: 12 | 15/23 |
| | Blindfolded black | Night jewels 1 | #200 | 4 total- 1x control (BLC1), 3x blindfolded (BLP1-3) | BLC1: 14, BLP1: 14, BLP2: 16, BLP3: 4 | 14/34 |
| | Blindfolded white | Chiffon white 4 | | 4 total- 1x control (WC1), 3x blindfolded (WP1-3) | WC1: 11, WP1: 15, WP2: 15, WP3: 8 | 11/38 |
| 2014 | Blindfolded extreme luminance green | Indian ivy 3 | #250 | 4x total- 2x control (GC1, GC2), 2x blindfolded (GP1, GP2) | GC1: 15, GC2: 11, GP1: 14, GP2: 12 | 26/26 |
| | Blindfolded extreme luminance brown | Espresso shot | | 4x total- 2x control (BC1, BC2), 2x blindfolded (BP1, BP2) | BC1: 15, BC2: 14, BP1: 14, BP2: 13 | 29/27 |
| | Blindfolded black | Night jewels 1 | #270 | 2x total- 1x control (WC2), 1x blindfolded (WP4) | WC2: 12, WP4: 6 | 12/6 |
| | Blindfolded white | Chiffon white 4 | | 2x total- 1x control (BLC2), 1x blindfolded (BLP4) | BLC2: 15, BLP4: 11 | 15/11 |

## Blindfolding

Following a pilot study, black acrylic paint (Royal Langnickel Essentials Acrylic Paint PNTA158 BLACK) was chosen as the most suitable method to occlude light from ocelli (Fig. 3.1), applied with a Royal Langnickel Sable Hair Detail Brush (Liner 5/ 0,0). Larvae were checked twice daily for signs of head capsule slippage (HCS). Individuals presenting signs of HCS were removed from the treatment arenas and placed into clear plastic 200 mL cups, containing only food material, in darkness. Following complete HCS, the ocelli of these individuals were re-painted and they were placed back into treatment arenas. The maximum time taken for complete HCS from beginning to end is 24 hours (Edmonds, 2010), but larvae had usually completed HCS 4-12 hours after removal from dowel. Larvae for non-blindfolded control treatments were a random sub-set from the same full-sib families (Table 3.1).

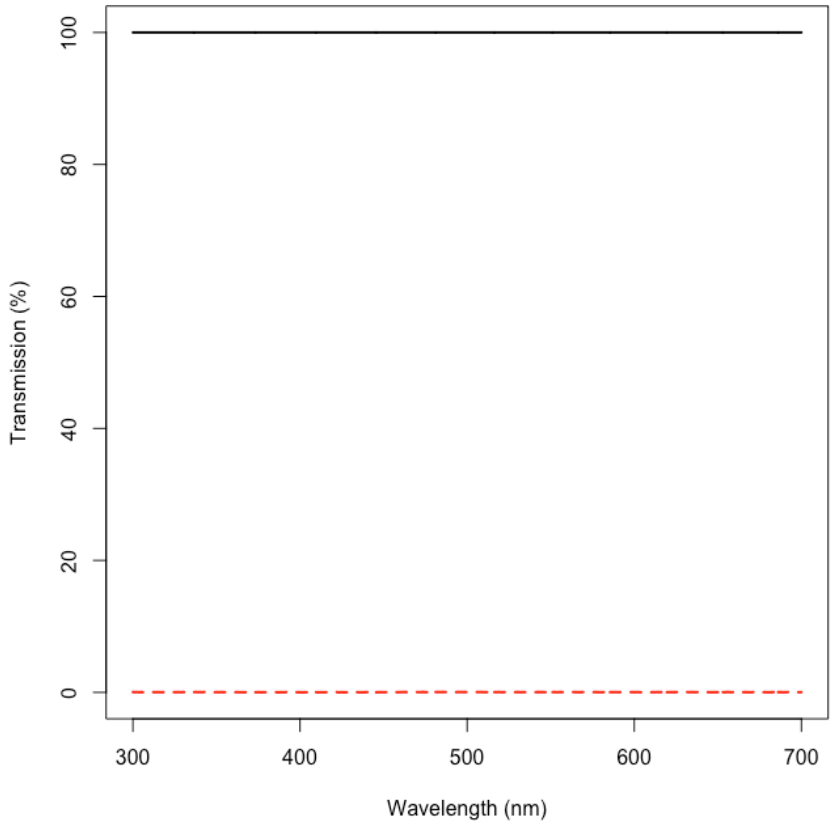


**Figure 3.2. Effectiveness of blindfolding at blocking light transmission.** Mean percentage of light transmitted through 2mm thick clear plastic when unpainted, representing ocelli of control larvae (black line), and painted with the same black acrylic paint used to blindfold larvae (dashed red line).

An indication of the effectiveness of the blindfolding treatment to completely block light from entering the ocelli, is provided by measurement of the light transmitted through painted and unpainted 2mm thick clear acrylic plastic squares (5 replicates), using a spectrophotometer (Fig. 3.2).

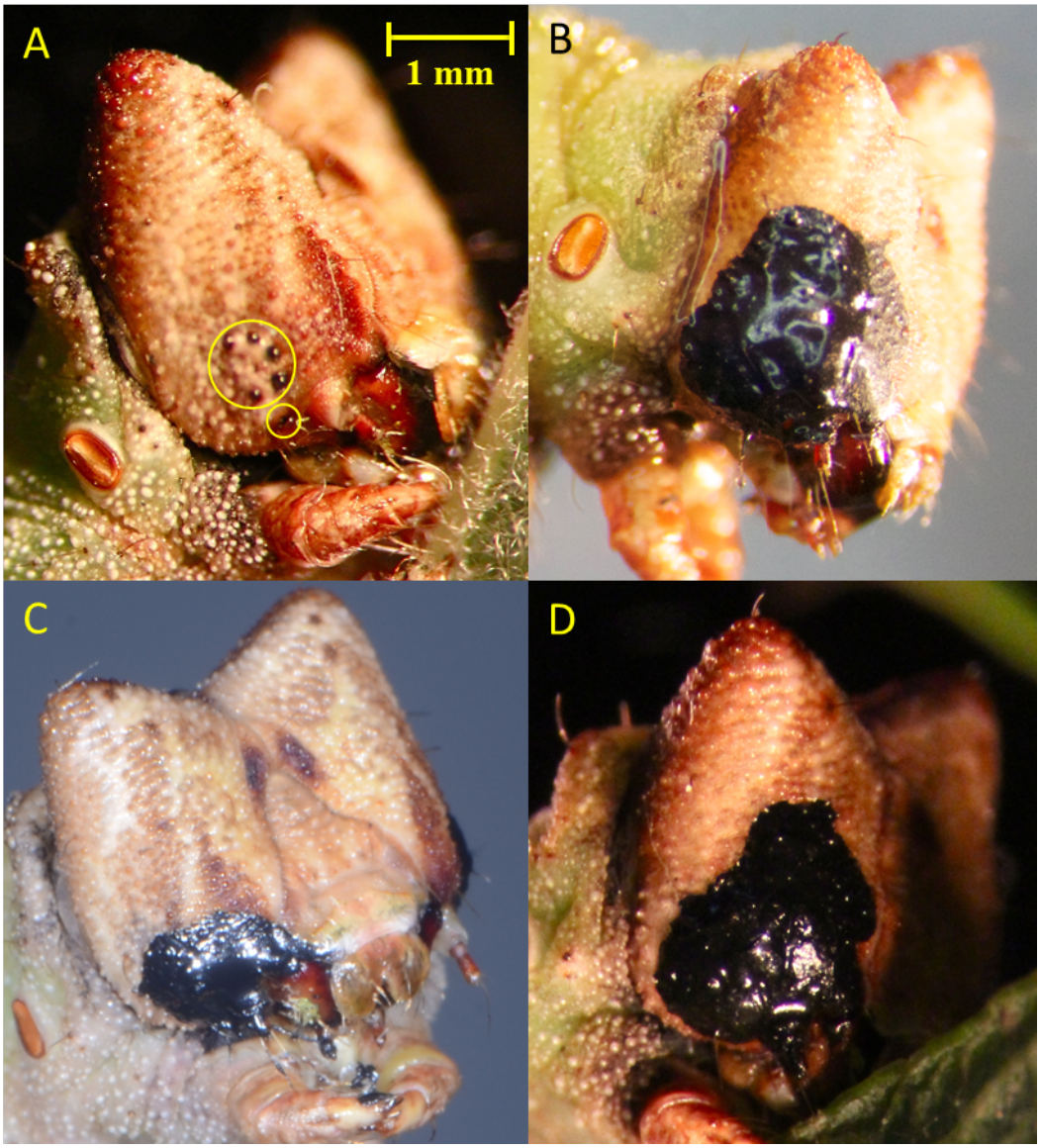


**Figure 3.1. Blindfolding *B. betularia* larvae.** (A) Control larva with ocelli circled and single 6th ventral ocellus circled independently. (B-D) Representative examples of blindfolded larvae with black acrylic paint covering 6 ocelli on each side of the head.

### Quantifying the colour response

The reflectance of final instar larvae (and painted dowels) was measured using an Ocean Optics USB2000 spectrophotometer, with a DH-2000 halogen deuterium light source. Spectra were recorded at 0.34 nm intervals from 177.7-881.13 nm and measured relative to a WS-1 reflectance standard using Overture (version 1.0.1). Larvae were cooled for several minutes prior to measurement to reduce movement. A total of six measurements were taken; three from the left and three from the right lateral surfaces of each individual, always recorded from the 3rd thoracic segment, and the 2nd and 6th abdominal segments. This was to prevent overlap in measurements, and because these segments showed no prominent markings. Reflectance spectra were reduced to 1 nm intervals within the 300-700 nm range (see

Fig. S3.2 for raw spectra) and processed to calculate stimulation of UV, SW, MW and LW cones for chromatic (green and brown) dowels and stimulation of double dorsal or DD cones for achromatic (black and white) dowels, following the same methodology described in Chapter 2. For brown and green treatments, as a simpler measure of colour, we calculated 'greenness' opponent channels MW/(MW+LW). For the achromatic (black and white) treatments, we analysed response to luminance only, using the blue tit double dorsal cone catch, as these cones mediate luminance vision (Campenhausen & Kirschfeld 1998; Osorio & Vorobyev 2005).

**JND analysis**

As a further measure of the larval luminance and chroma response under blindfolding, we modeled the ease with which an avian predator might discriminate between larvae and their corresponding dowel using just noticeable differences (JND) (see Vorobyev and Osorio, 1998 for equations). For chromatic contrasts (green and brown larvae vs. dowels), we used spectral sensitivities of the blue tit (*Cyanistes caeruleus*) using relative cone ratios of SW=0.7111; MW=0.9926; LW=1.0 and UV=0.3704 (Hart *et al.*, 2000), with a Weber fraction of 0.05, and idealized irradiance (D65). To model luminance JNDs between black and white larvae and dowels, we used blue tit double dorsal (DD) cones. JND <1.00 indicate that two stimuli are indiscriminable; stimuli differing by 1-3 JND units are only discriminable under good viewing conditions; and stimuli showing values above this should be distinguishable with increasing ease (Stevens *et al.*, 2015).

**Statistical analysis**

Treatment effects (dowel colour and blindfolding) on larval colour (greenness), larval luminance (DD), and avian visual discrimination (JND) were assessed via linear models (LMs). Data were log transformed where distributions differed from normal. All statistical analyses were performed in R version 3.3.2 (R Core Team, 2014).

## RESULTS

### Blindfolded response to luminance

As expected (from Chapter 2), final instar *B. betularia* larvae reared on white dowels were significantly brighter than those reared on black dowels ($F_{1, 127}$ =177.4, $P$<0.0001; Fig. 3.3), but there was no significant effect of blindfolding on the luminance of larvae from black or white treatments, ($F_{1, 127}$ = 0.28, $P$=0.6). Larvae from black treatments were much more similar in luminance to their corresponding dowels, with a negligible difference in DD cone catch values, than larvae from white treatments, which showed a DD cone catch difference of 0.3 from their dowels (Fig. 3.3).

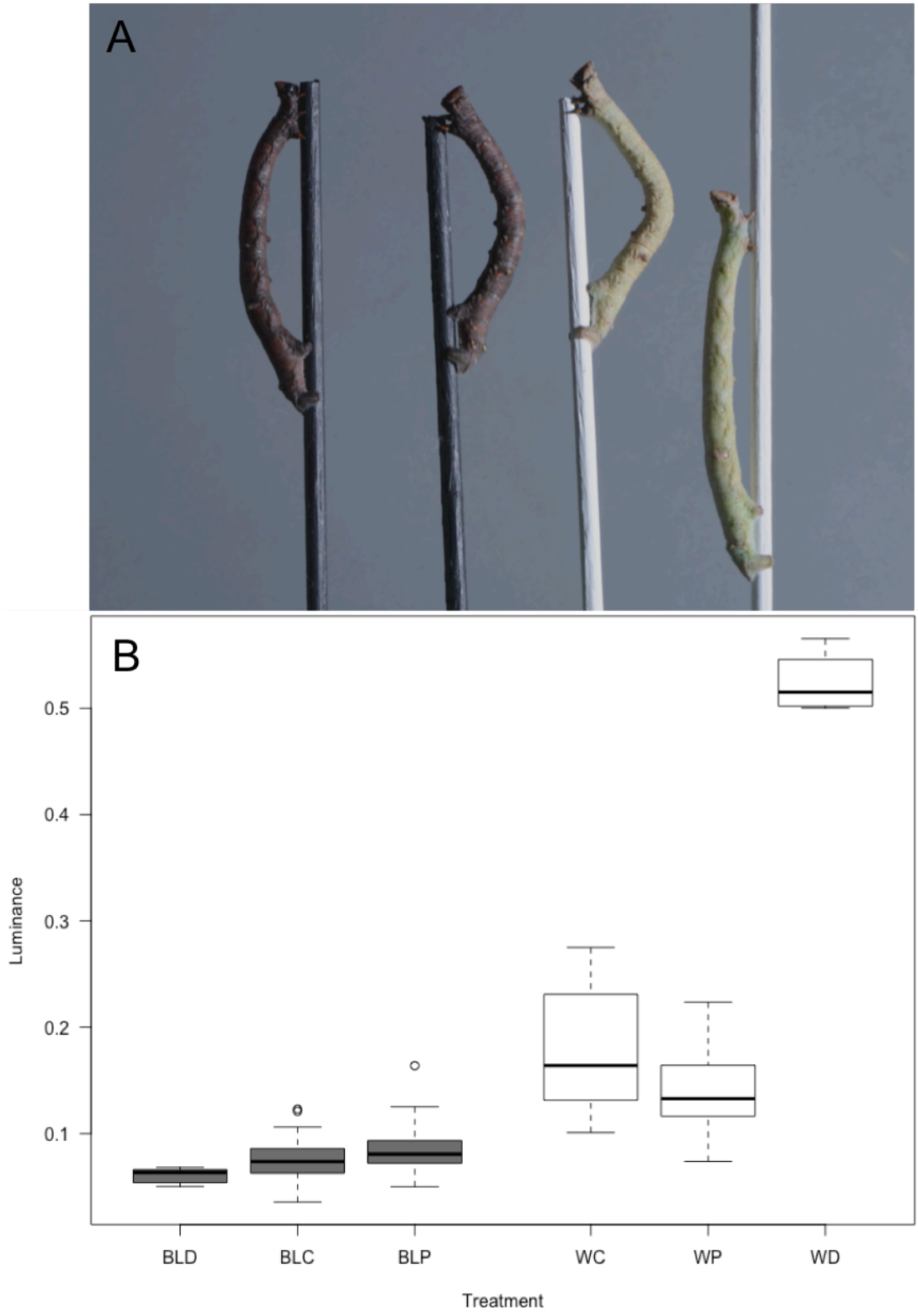


**Figure 3.3. Response of blindfolded *B. betularia* larvae to black and white dowels.** (A) Representative final instar larvae on corresponding luminance dowels (leftmost of each pair is blindfolded, rightmost is non-blindfolded control). (B) Luminance of final instar larvae and dowels. BLD= black dowel; BLC= black control larvae; BLP= black blindfolded larvae; WC= white control larvae; WP= white blindfolded larvae; WD= white dowel. Boxes represent median (midline) ± interquartile range (IQR), whiskers represent IQR ± (1.5 x IQR), open circles= outliers.

**Blindfolded response to chroma**

Brown and green larvae differed to each other in avian colour space, but blindfolded and control larvae from both brown and green colour treatments overlapped in colour space (Fig. 3.4). Green and brown larvae were a similar distance from their corresponding dowels (Fig. 3.4).

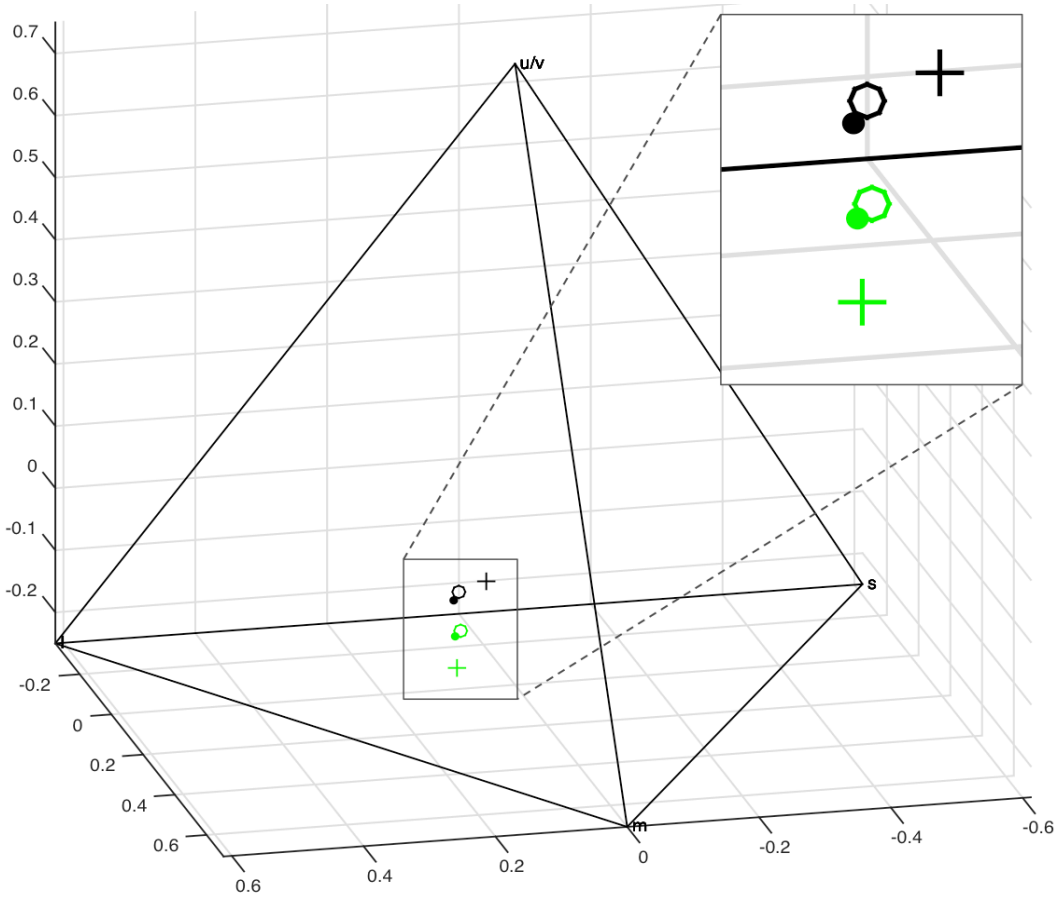


**Figure 3.4. The response of blindfolded *B. betularia* larvae to green and brown dowels in avian colour space.** The distribution of final instar *B. betularia* larvae and their corresponding dowels within the ultraviolet-sensitive (UVS) avian tetrahedral colour space when viewed by a blue tit, *Cyanistes caerulys* under bright daylight conditions. Black= brown treatments and green= green treatments, where crosses represent dowels, open circles represent control larvae, and filled circles represent blindfolded larvae. The plot illustrates the stimulation of the short (S), medium (M), long (L), and UV (U/V) wavelength-sensitive photoreceptors and is shown from the MW plane.

Larvae reared on green dowels had significantly higher greenness values than larvae from brown treatments ($F_{1,\ 169}$ =457.8, $P$<0.0001; Fig. 3.5). Blindfolding had no significant effect on larvae greenness for green or brown treatments ($F_{1,\ 169}$ =0.55, $P$=0.5), and the distribution of greenness was almost identical between blindfolded and control larvae across both treatments (Fig 3.5). Brown larvae were more similar in colour (greenness) to brown dowels (showing negligible differences), than green larvae to green dowels, which were 0.1 greenness units lower than their dowels (Fig. 3.5).

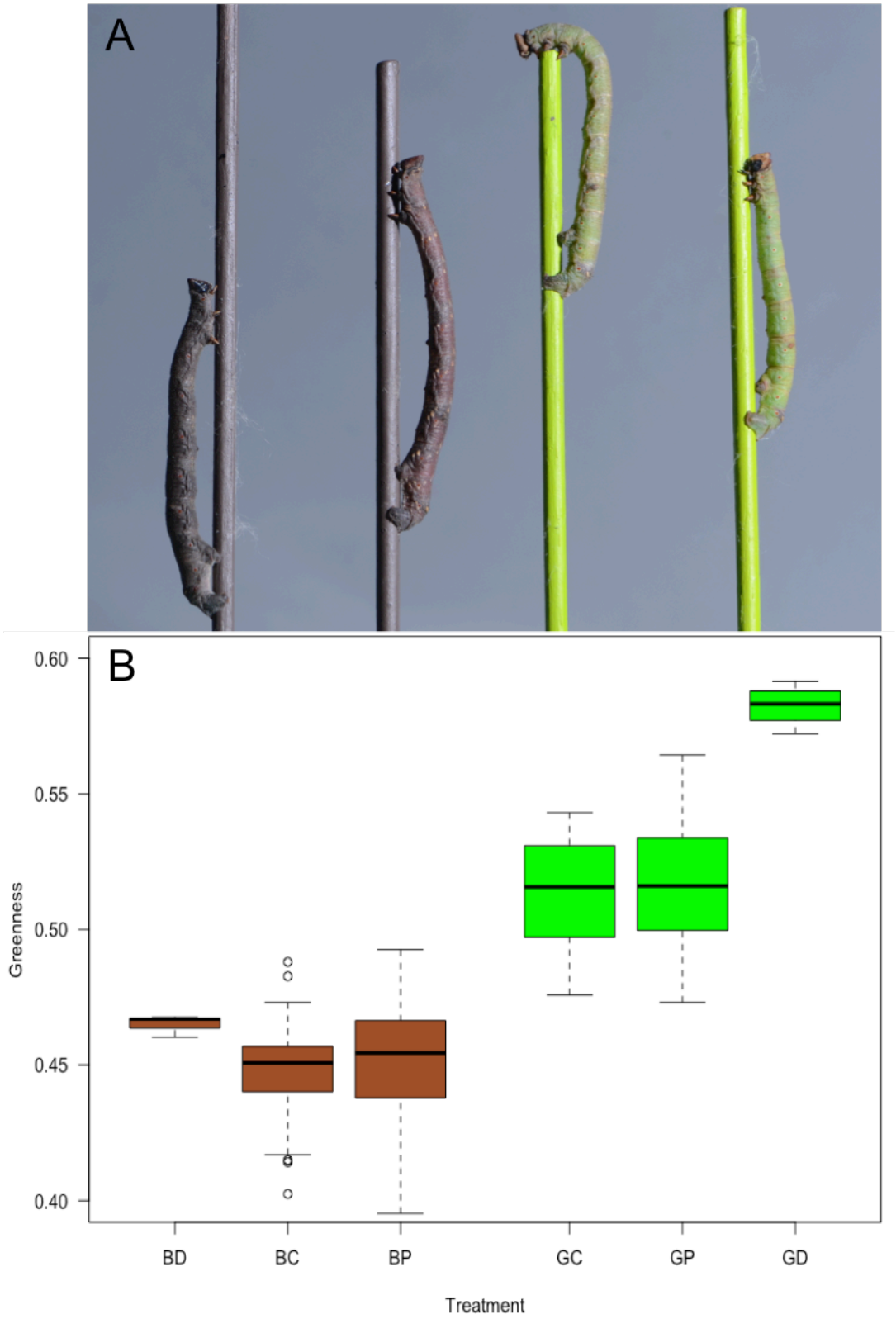


**Figure 3.5. Response of blindfolded *B. betularia* larvae to brown and green dowels. (**A) Representative final instar larvae on corresponding chroma dowels (outermost larvae are blindfolded, inner two are not). (B) Greenness boxplots of dowels (BD, brown dowel; GD, green dowel) and final instar larvae (BC, brown control; BP, brown blindfolded; GC, green control; GP, green blindfolded). Boxes represent median (midline) ± interquartile range (IQR), whiskers represent IQR ± (1.5 x IQR), open circles= outliers.

Larvae reared on green dowels were significantly brighter than larvae from brown treatments ($F_{1, 169}$ =12.36, $P$<0.0001; Fig. S3.3), although the difference between larvae from brown and green treatments was smaller for luminance (~0.05) than for greenness (~0.3) (Figs. 3.5 and S3.3). Blindfolding did not affect larvae luminance ($F_{1, 169}$ =0.775, $P$=0.4). As with greenness, the difference in luminance between larvae and their corresponding dowels was greater for the green treatment (Fig. S3.3).

**Ability to match dowels (JND analysis)**

Black larvae were able to match the luminance of black dowels more closely than white larvae could match the luminance of white dowels ($F_{1, 127}$=165.9, $P$<0.0001), with JND ~5 for black larvae and ~25 for white larvae (Fig. 3.6). Blindfolding did not affect how well black or white larvae could match their dowels ($F_{1, 127}$= 2.64, $P$= 0.1; Fig. 3.6). Brown larvae were able to match the chroma of brown dowels more closely than green larvae matched the chroma of green dowels, as shown by significantly lower JND for brown larvae vs. brown dowels (~7), than green larvae vs. green dowels (~14), ($F_{1, 169}$= 79.14, $P$<0.0001). There was no effect of blindfolding for either brown or green larvae on JND between larvae and dowels ($F_{1, 169}$= 1.01, $P$= 0.3).

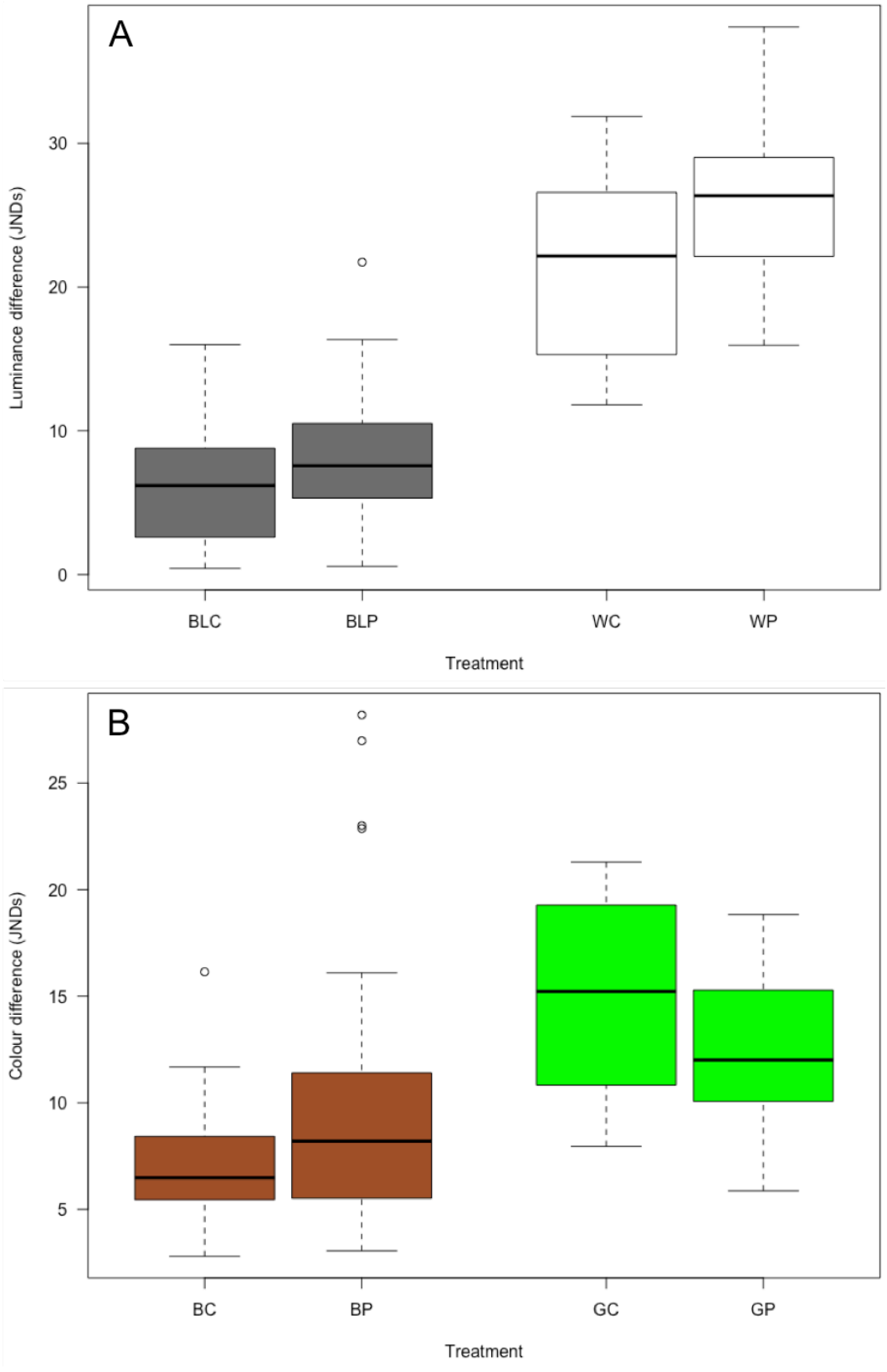


**Figure 3.6. Discriminable differences between *B. betularia* larvae and corresponding dowels.** (A) Luminance differences between black and white larvae from control and blindfolded treatments and corresponding dowels. (B) Colour differences between brown and green larvae from control and blindfolded treatments and corresponding dowels. Treatments are: BLC (black control), BLP (black blindfolded), WC (white control), WP (white blindfolded), BC (brown control), BP (brown blindfolded), GC (green control), GP (green blindfolded). Boxes represent median (midline) ± interquartile range (IQR), whiskers represent IQR ± (1.5 x IQR), open circles= outliers.

**DISCUSSION**

Blindfolded larvae were able to change colour to match artificial twig backgrounds varying in both chroma and luminance, to the same extent as control, non-blindfolded larvae. This suggests that *B. betularia* larvae are processing visual cues using functional photoreceptor cells located outside the ring of ocelli. It is possible that ocelli may not even function in the colour change response, particularly if they are limited in visual capability, as suggested. *B. betularia* caterpillars remain motionless during daylight hours, holding a characteristic twig-posture in which the head is relatively far removed from the twig perch (Edmonds, 2010), where photoreceptors in the skin may convey more accurate colour information for closer background matching.

Extraocular photoreceptors (EOPs) containing photopigments play a major role in circadian rhythm (Land & Nilsson, 2002), where they are often assembled into specialised light-sensitive organs, such as the Hofbauer-Buchner eyelet (H-B eyelet) in *Drosophila* (Hofbauer & Buchner, 1989; Veleri *et al.*, 2007) and the pineal eye in reptiles, amphibians, and fish (Mrosovsky & Tress, 1966; Korf *et al.*, 1981; Tosini, 1997; Herrera-Perez *et al.*, 2015). Alternatively, photopigments used for circadian rhythm may be distributed in the dermis and deeper neural tissues, such as the brain and abdominal ganglia of vertebrates and invertebrates (Wilkens & Larimer, 1976; Wolken & Mogus, 1979; Shimotsu *et al.*, 2010; Kokel *et al.*, 2013). We have not yet determined the exact location of EOPs in *B. betularia*, but it is likely, based on the uniform colour change across the larvae dermis, that they are located in dermal tissue. Dermal photoreceptors have been reported in *Drosophila* larvae and a species of pond snail, *Lymnaea stagnalis*, where they function in sensing achromatic light and therefore exposure to aid in predator avoidance (Chono *et al.*, 2002; Xiang *et al.*, 2010). Other functions of EOPs associated with predator avoidance include regulating the release of stinging cells in cnidarians (Plachetzki *et al.*, 2012), and guiding colour change, as our findings suggest in *B. betularia* larvae.

The most recognised example of colour change through dermal photoreception is in cephalopods (Kingston *et al.*, 2015; Ramirez & Oakley, 2015). This system utilises chromatophores to achieve rapid (<5 seconds) and localised colour camouflage, despite the fact that these animals are colour-blind (Messenger, 1977; Mathger *et al.*, 2006). Frogs and fish are also able to match their backgrounds rapidly using dermal chromatophores (Ramachandran *et al.*, 1996; Kelman *et al.*, 2006; Kindermann &

Hero, 2016). Moorish geckos show a relatively slower response to luminance, with blindfolded individuals able to darken or lighten their skin to match the luminance of their substrate using dermal melanophores (Vroonen *et al.*, 2012; Fulgione *et al.*, 2014). As far as we are aware, our findings are the first that demonstrate EOP-mediated response to chroma in addition to brightness, without using chromatophores.

Colour change in *B. betularia* larvae is comparatively slower than that effected by chromatophores, taking days to weeks to successfully match a background (Edmonds, 2010) Slow colour changes from visual cues have rarely been reported, but occur in a species of crab spider, *Misumena vatia*, which deposits ommochrome pigment granules in epidermal cells to achieve a range of colours (Insausti & Casas, 2008; Insausti *et al.*, 2012). The larvae of two hawk moth species, *Laothoe populi* and *Smerinthus ocellata*, change colour over a number of days to match changes in brightness and colour of their visual backgrounds (Grayson & Edmunds, 1989). In both of these examples, it is not known whether the proposed visual signals that mediate colour change are processed within the retina or outside it.

Although the detail of the colour change mechanism in *B. betularia* is currently unknown, it is likely that visual information on colour is transmitted neurally and/or hormonally to signal appropriate pigment production. There may even be a local neural network in the dermal tissue, similar to chromatophores, which can process colour information and signal pigment deposition via a feedback mechanism. Opsins are key photopigments in colour vision and many studies on extraocular photoreception have located opsin genes expressed outside of the retina (Shiraki *et al.*, 2010; Fulgione *et al.*, 2014; Kingston *et al.*, 2015; Ramirez & Oakley, 2015). However, in *Drosophila spp.*, the gene *tan* encodes an enzyme that is required in both photoreceptor function and melanin pigmentation, which could potentially process visual information and regulate melanism in dermal cells (True *et al.*, 2005). Gene or protein expression of photopigments and other visual genes may provide further insight into the first stage of the mechanism, and hormone analyses could indicate which hormones, if any, trigger the colour change in *B. betularia.* In the stick insect, *Carausius morosu*s, colour change is affected by visual stimulation (Buckmann, 1977). Buckmann (1977) investigated the process that occurs between phototransduction and colour change by painting over the eyes of individuals with black varnish, and found that these animals synthesised ommochrome pigments from

tryptophan to darken their integument. It is assumed that the rate of ommochrome synthesis is under hormonal control, as with colour determination in most insects (Gade *et al.*, 1997). Ommochrome synthesis in the tobacco hornworm moth is controlled by juvenile hormone (Hori & Riddiford, 1982). These results lead us to believe that if *B. betularia* larvae were only using their eyes for colour change, they would darken similarly to the stick insects in Buckmann's (1977) study.

Extraocular photoreception has previously been reported in Lepidoptera, but not for the purpose of camouflage. Brain photoreceptors in the larvae of two moth species, *Antheraea pernyi* and *Bombyx mori*, regulate circadian rhythm (Tanaka, 1950; Shimizu, 1982). The genitals of swallowtail butterflies, both male and female, contain photoreceptors used in mating and oviposition (Arikawa & Miyako-Shimazaki, 1996). However, our findings in *B. betularia* appear to be the first example of colour change mediated by EOPs in Lepidoptera, perhaps even insects, with the possible exception of Poulton (1892) in *Aglais urticae* and a later study by Angersbach (1975) in *Pieris brassicae*. Both authors occluded the ocelli of the late stage butterfly larvae with black paint and found that the pupae still melanised according to the colour of the background the larvae were last exposed to. It was therefore suggested that EOPs existed in the skin of *A. urticae* (Poulton, 1892), whereas Angersbach (1975) proposed that extra-retinal photoreception was attained through a dorsal photoreceptor located on the surface of the head of *P. brassicae* caterpillars.

The JND between larvae and dowels were much higher than the discrimination threshold (1) for all treatments, especially white and green. High absolute JND were also found in a similar study on background matching in crabs, where stimuli were more extreme to what individuals would be exposed to naturally (Stevens *et al.*, 2014b). High JND between larvae and dowels in our experiment are also not surprising for the same reason, especially considering the high luminance of the white dowels and high greenness of the green dowels. The JNDs were also calculated under bright light conditions, whereas in a natural setting, light levels would vary during predator/prey interactions, and so the threshold discrimination may be higher under poorer light conditions. As discussed in chapter 2, background colour alone is not responsible for crypsis in *B. betularia*. As well as matching the background colour of their environments, larvae also use a combination of pattern and posture to

match visually complex environments, which were not taken into account in our JND analysis.

## CONCLUSIONS

Our results show that larvae of the peppered moth are able to process visual cues to closely match the colour and luminance of their background without using their ocelli. This is, to our knowledge, the first example of extraocular photoreception that is able to discriminate between different wavelengths of light, i.e. colour vision, and the first evidence of colour change in lepidopteran larvae mediated through EOPs. Future work should aim to explore the visual mechanism, through molecular and physiological means such as visual genes and neural networks.

# Chapter 4

## Behavioural background matching and extraocular photoreception in *Biston betularia* larvae

**ABSTRACT**

Environments are rarely uniform over space and/or time. To avoid detection, animals may achieve effective camouflage in visually heterogeneous environments either by changing colour or pattern to match their background, and/or by choosing an appropriate background that matches their own colour pattern. We have previously shown that peppered moth (*Biston betularia*) larvae change colour to match the luminance and chroma of the twigs they rest upon. We hypothesised that larvae would rest upon backgrounds that better matched their own colour and found that this was true. Surprisingly, larvae that had been blindfolded were also found to rest more frequently on matching backgrounds compared with non-matching backgrounds. It is likely that larvae are processing visual information about the colour of their backgrounds through dermal photoreceptors, and suggests additional involvement of the central nervous system. The colour change in *B. betularia* larvae is slow (14-21 days), and sometimes imperfect. Therefore, we suggest that choosing matching backgrounds is less costly than slow colour change in variable environments and may protect larvae from predation during slow colour change. This is one of the first examples of behavioural background matching using extraocular photoreception.

**INTRODUCTION**

Predation is a key selective pressure that has driven a wide range of defences, including visual camouflage in a diverse range of animal taxa (Stevens & Merilaita, 2009). Arguably, the most common form of visual camouflage is background matching (Thayer, 1909). The visual environment in which an animal lives is rarely constant over space and time. Therefore, in heterogeneous environments, animals may show phenotypic plasticity in morphology and/or behaviour. Colour change can enable individuals to match the colour patterns of their current environment (Grayson & Edmunds, 1989; Kang *et al.*, 2016; Polo-Cavia *et al.*, 2016). Additionally, animals may choose to rest against matching backgrounds or in positions that retain crypsis, known as behavioural background matching (Garcia & Sih, 2003).

Habitat choice behaviour is often observed in animals with fixed genetic morphs; for example: in a release and relocate experiment, Sandoval (1994b) found that the grey and red morphs of the walking stick, *Timema cristinae*, rested on twigs in higher

proportions than the green morph and that the green morph rested on leaves more than other morphs. Predation experiments showed that this behaviour reduces detection by avian predators. Another release and recapture experiment showed similar results with the limpet, *Lottia digitalis*, which returned back to habitats that matched their shell colour (Byers, 1989). Pygmy grasshoppers (Ahnesjö & Forsman, 2006), and pacific tree frogs (Morey, 1990) also chose to rest on backgrounds that better matched their own colour. Some animals choose habitats that increase crypsis of body pattern, rather than colour. For example, the least killifish prefers to rest against horizontal stripes, which match the stripes on its body, rather than mismatching vertical stripes (Kjernsmo & Merilaita, 2012). Similar behaviour has been observed in several species of geometrid moth, which orient themselves in positions on tree trunks to minimise their outline (Kang *et al.*, 2012; Kang *et al.*, 2013). The two fixed colour pattern morphs of the Eastern red-backed salamander adopt different postures in response to different attack rates, a behaviour that is likely to contribute to the maintenance of this polymorphism (Venesky & Anthony, 2007).

Animals may combine colour change with habitat choice to further increase crypsis. Flounders change colour in 1-3 seconds, which is a relatively fast response, but can only produce a limited repertoire of colours. In background choice trials, they have been found to avoid bright colours, instead preferring to rest on neutral-coloured backgrounds with little patterning, to achieve a closer background resemblance. This behaviour has likely arisen because bright colours, such as purple and yellow, are more difficult to match (Tyrie *et al.*, 2015). The cost of colour change could be another reason for behavioural background matching observed in species that are capable of rapid colour change. It is known that changing colour incurs metabolic costs in colour-changing fish and newts (Rodgers *et al.*, 2013; Polo-Cavia & Gomez-Mestre, 2017). Choosing backgrounds avoids paying the cost associated with colour change but may present an opportunity cost; for example, limited food resources in that habitat (Ruxton *et al.*, 2004).

An animal may also use a combination of plastic colour change and behavioural habitat choice if they change colour slowly, as during this time they are vulnerable to predation if the degree of matching is not close enough to remain undetected (Fairchild & Howell, 2004). Some species of flatfishes exhibit a relatively slow colour change (1-3 days), and so prefer to settle on substrates of colours and patterns similar to their own to increase their match to the substrate (Sumner, 1911; Ryer *et*

*al.*, 2008). Some species of lepidopteran larvae also show gradual colour change, such as the peppered moth (chapters 2 and 3; Noor *et al*., 2008), and eyed and poplar hawkmoths (Grayson & Edmunds, 1989) in response to visual backgrounds. However, there is no experimental evidence of behavioural background matching to increase concealment in these species.

*Biston betularia* caterpillars change colour to match the twigs upon which they rest to avoid predation (Noor *et al*., 2008), and are able to do this without the use of their ocelli (chapter 3). This change occurs comparatively slowly, taking 2-4 weeks to produce a complete colour change (chapter 2), and during this time, the larvae are likely vulnerable to predation. We designed a series of experiments, following on from the colour response experiments (see chapters 2 and 3), to explore whether *B. betularia* choose microhabitats that match their own colour and luminance, and whether they can do so without the use of their ocelli. We tested the following hypotheses:

1) Larvae choose to rest on branches that most closely match their own colour (both for isoluminant chroma and extreme colour contrasts).

2) Blindfolded larvae, with obscured ocelli, are able to select between microhabitats differing in luminance and colour to the same extent as non-blindfolded control larvae.

## METHODS

### Rearing and choice chambers

*Biston betularia* larvae used for these experiments were reared to final instar as part of the colour response experiments described in chapters 2 and 3. We used two different designs of choice chamber; crossed dowels (Fig. 4.1) and horizontal dowels (Fig. 4.2). Crossed dowel choice chambers consisted of clear plastic boxes measuring 70 x 70 x 80 mm (length x width x depth- including lid). Inside each chamber were two dowels measuring 112 mm crossed over: one green and one brown. On the floor of the chamber, a mark was made, equidistant from either dowel (Fig. 4.1). Larvae were placed on the mark, facing neither dowel. Cylindrical choice chambers were clear plastic bottles (210 mm long x 60 mm diameter) (Fig. 4.2) Inside each chamber was a 200 mm dowel, painted half brown and half green with the same colours larvae were reared on (see Table 4.1 and chapter 3 for dowel colours). Two trials were conducted per individual, in which the orientation of the dowel relative to the

chamber was switched, to control for any innate larvae direction preference or lighting effects. The rationale for these two designs was to ensure that larvae were actually selecting habitats, as the crossed design may have made it more difficult for larvae to perceive both dowel colours.

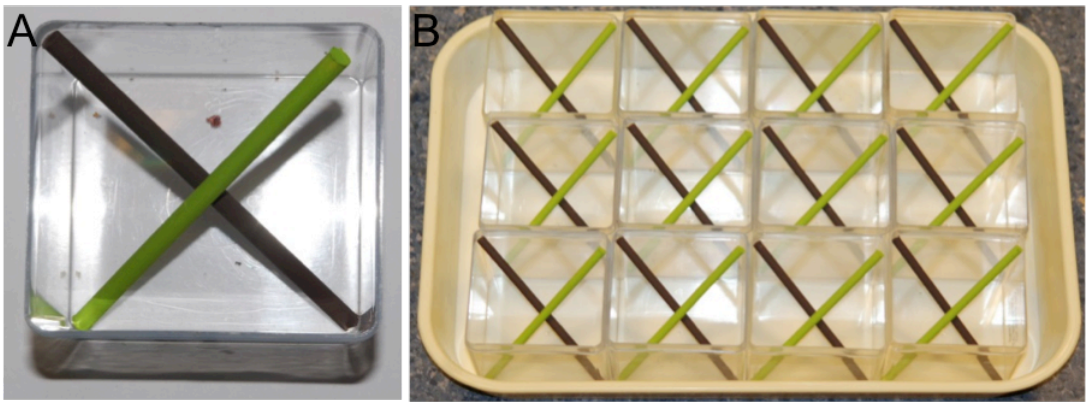


**Figure 4.1. Crossed dowel choice chamber used for microhabitat choice experiments.** (A) Close up view of choice chamber. (B) Example of how choice chambers were set up. Both examples of chambers contain extreme luminance dowels.

Prior to placement in choice chambers (crossed and horizontal), larvae were gently poked three times along their dorsal surface with tweezers: this was found to be an effective way of simulating predation (Skelhorn & Ruxton 2012). Experiments were conducted using 12 individuals at a time (Fig. 4.1) in a Sanyo Versatile Environment Test Chamber (model MLR-351) on light level 4 (15,000 lx). Each trial consisted of larvae from different treatments (colour and blindfolding/control). This was partially due to random selection of individuals, but also because larvae had to have reached final instar, and growth rates of individuals differed within treatments. Larvae ID describing treatment (and individual for horizontal dowel trials) was placed on the bottom of each chamber and verified after resting position was recorded. If a larva was resting across both dowels, the dowel that the largest proportion of the larva was resting on was coded. If larvae were on the floor of the chamber, they were excluded from the trial.

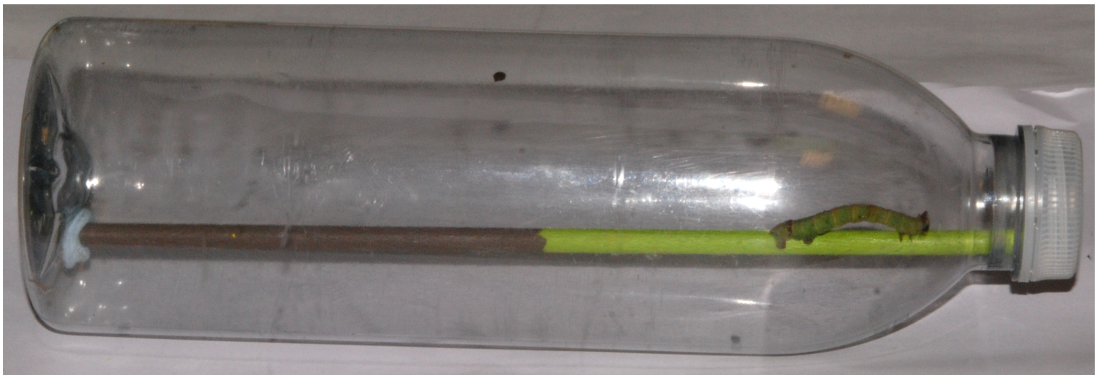

**Figure 4.2. Choice chamber for horizontal microhabitat experiments.** Choice chamber shows one of two dowel positons; other position has green end of dowel at the base of the chamber.

In preliminary tests of this experiment, larvae were left in the choice chamber for 30 minutes before their resting position was coded. It was found that a high proportion of larvae were still on the floor of the chamber. We therefore increased the time inside the chamber to 12h (5 hours light, 7 hours darkness), taking into consideration the sedentary behaviour of the larvae, and therefore simulating a situation more typical in nature. Two types of green and brown dowel contrasts were used to test responses to chroma and luminance independently (Table 4.1).

**Choice experiments with isoluminant dowels**

To test for behavioural choice in response to colour and not luminance, we used control (non-blindfolded) final instar larvae that had been reared on either isoluminant green, or brown dowel colours (chapter 2). These dowels differed in colour but were similar in luminance (Table 4.1; chapter 2, Fig. 2.2). The same colours that larvae had been reared on were used in crossed dowel microhabitat choice chambers.

**Choice experiments with extreme luminance dowels**

To test for luminance as an additional cue to colour, and to evaluate the effect of obscuring ocular vision on behavioural background matching, we used control and blindfolded final instar larvae that had been reared on ‘extreme brown’ or ‘extreme green’ dowels (chapter 3). These dowels differed in both chroma and luminance (Table 4.1; chapter 2, Fig. 2.2). The same coloured dowels that larvae had been reared on were used in both crossed and horizontal dowel choice chambers.

**Table 4.1.** Details of experiments and related hypotheses

| Experiment | Dowel paints | Family ID | Sample size (*n*) | Hypothesis |
|---|---|---|---|---|
| i. crossed dowels | **Isoluminant-**<br>Brown: Wild mushroom 1<br>Green: Indian ivy 2 | 270 | Brown: 54<br>Green: 65 | 1a) Larvae rest on chroma that matches their body colour more frequently than would be observed by chance. |
| ii. crossed dowels | **Extreme luminance-**<br>Brown: Espresso Shot<br>Green: Indian Ivy 3 | 250 | Brown control: 62<br>Brown blindfolded: 60<br>Green control: 64<br>Green blindfolded: 59 | 1b) Larvae rest on colours that match their body colour more frequently than would be observed by chance.<br><br>2) Blindfolded larvae are able to select matching microhabitats to the same extent as control larvae. |
| iii. horizontal dowels | **Extreme luminance-**<br>Brown: Espresso Shot<br>Green: Indian Ivy 3 | 280 | Brown control: 33<br>Brown blindfolded: 35<br>Green control: 38<br>Green blindfolded: 33 | |

## Statistical analysis

To test whether larvae rested on a) isoluminant or b) extreme luminance dowels that matched their own colour more often than would be observed by chance, we performed two-tailed binomial tests on the number of successful matches per number of trials. The frequency that a larva would be resting on a particular dowel colour by chance was set at 0.5. To test the effects of blindfolding, treatment and dowel position on dowel colour choice, we performed generalised linear models (family= binomial) with blindfolding, treatment and dowel position as predictors of success (0 or 1). All statistics were performed using R version 3.3.2 (R Core Team, 2014).

## RESULTS

### Isoluminant crossed dowels

Brown larvae chose to rest on brown dowels significantly more than would be observed by chance, (two-tailed binomial test, $P<0.001$, $n= 54$) with a frequency of ~0.75 (Fig. 4.3). Green larvae rested on green and brown dowels equally, with a frequency of ~0.5 (Fig. 4.3) and this did not deviate from what would be observed by chance (two-tailed binomial test, $P=$ ns, $n= 65$).

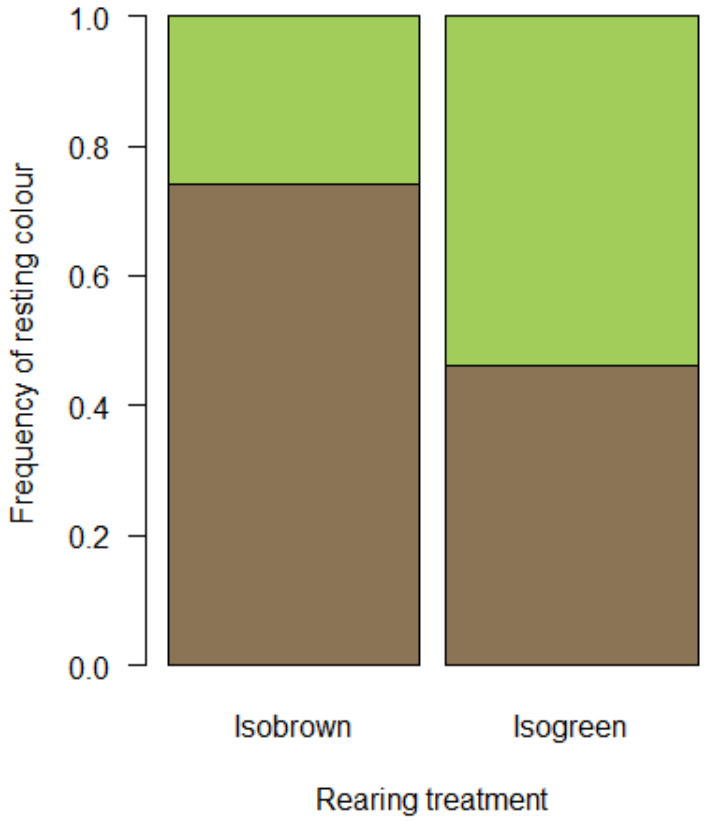


**Figure 4.3. Resting background chroma choice by *B. betularia* caterpillars.** Mean frequency of final instar *B. betularia* caterpillars reared on isoluminant brown (*n*= 54) or isoluminant green (*n*= 65) dowels found on each dowel colour (brown or green) 12 hours after being introduced into crossed dowel individual choice chambers.

### Extreme luminance dowels and blindfolding

Brown control and blindfolded larvae chose to rest on brown dowels significantly more than green dowels both in the crossed and horizontal dowel experiments (Table 4.2), with resting frequencies for blindfolded and control larvae in both experiments at ~0.8 on brown dowels (Fig. 4.4). Green control and blindfolded larvae rested on green dowels significantly more than brown dowels in both experimental chamber designs (Table 4.2). Resting frequencies on green dowels for green control and blindfolded larvae were slightly lower at ~0.7 (Fig. 4.4) than for brown larvae on brown dowels.

**Table 4.2. Background choice of *B. betularia* larvae in contrasting colour (extreme luminance) trials.** Two-tailed binomial tests of successful trials (success indicates matching colour chosen) with extreme brown and extreme green control and blindfolded larvae in crossed and horizontal dowel choice chambers.

| Choice chamber | Larvae type | *n* (success) | *n* (trials) | *P* |
|---|---|---|---|---|
| Crossed dowel | BC | 45 | 60 | 0.00013 *** |
| | BP | 41 | 56 | 0.00069 *** |
| | GC | 40 | 59 | 0.00864 ** |
| | GP | 35 | 51 | 0.01097 * |
| Horizontal dowel | BC | 26 | 34 | 0.00294 ** |
| | BP | 28 | 34 | 0.00020 *** |
| | GC | 29 | 37 | 0.00075 *** |
| | GP | 23 | 32 | 0.02006 * |

In the crossed dowel design, the colour of larvae had a significant effect on the colour of dowel they chose to rest upon (GLM: z=6.161, $P<0.001$). There was no effect of blindfolding (z=0.220, *P*= ns), or any interaction between blindfolding and larvae colour (z=-0.093, *P*= ns). In the horizontal dowel design, the colour of larvae also had a significant effect on the dowel colour they chose to rest upon (GLM: z= 5.26, $P<0.001$) and no effects were found of blindfolding (z=0.63, *P*= ns), or position of the dowel (z=0.31, *P*= ns).

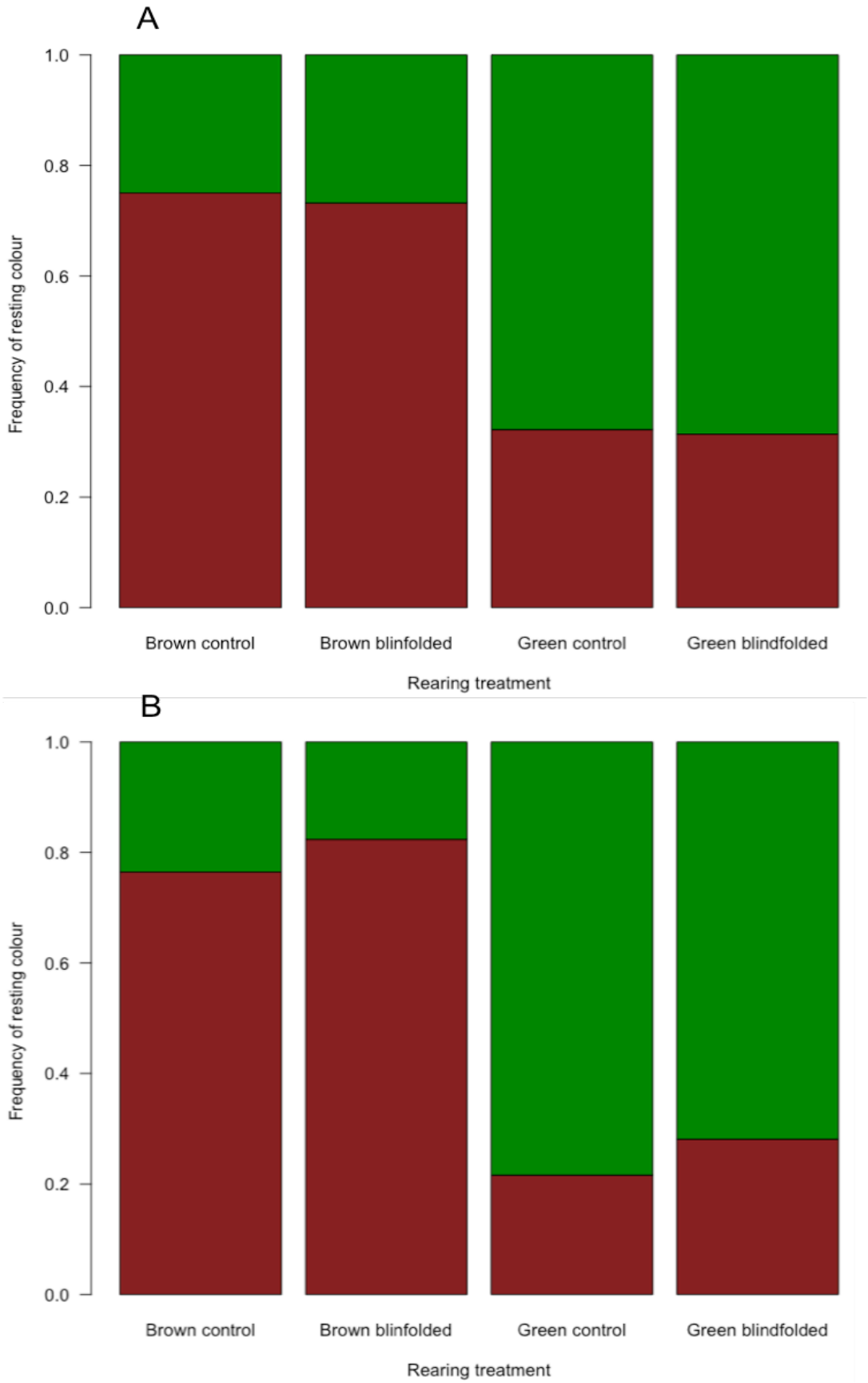


**Figure 4.4. Choice of resting background colour by blindfolded caterpillars.** Mean frequency of final instar *B. betularia* control and blindfolded caterpillars found on each dowel colour (contrasting luminance green or brown). Individual larvae from blindfolding experiments were placed in either crossed dowel arenas (A), or horizontal dowel arenas (brown dowel end at the back) (B) and their resting choice was recorded after 12 hours.

## DISCUSSION

*B. betularia* larvae were found to rest on dowels that more closely matched their own body colour, when presented with green and brown dowels that differed in hue and luminance. Surprisingly, this result still held in larvae with obscured ocelli, suggesting that extraocular photoreception is involved in behavioural background choice in *B. betularia*. When presented with a choice of green or brown dowels that differed only in hue and not luminance, only brown larvae chose to rest on brown dowels, whereas green larvae chose to rest on both equally.

Behavioural background matching to maximise crypsis is common in animals with fixed phenotypes; for example, peppered moth adults, limpets, stick insects, and frogs choose backgrounds that more closely match their own colour (Kettlewell, 1956; Byers, 1989; Sandoval, 1994b; Wente & Phillips, 2003). Animals that are capable of colour change also sometimes choose backgrounds to prevent visual detection in heterogeneous environments, such as larval newts, flatfish, and salamanders (Garcia & Sih, 2003; Ryer *et al.*, 2008; Tyrie *et al.*, 2015; Polo-Cavia & Gomez-Mestre, 2017).

Although colour change is advantageous for camouflage in heterogeneous environments, it incurs physiological costs (Rodgers *et al.*, 2013; Skold *et al.*, 2013; Polo-Cavia & Gomez-Mestre, 2017). In rapid colour changing systems, neuroendocrine control of chromatophores is highly costly (Aspengren *et al.*, 2006; Aspengren *et al.*, 2009). We have not directly measured the cost of colour change in *B. betularia* larvae, but have observed that this comparatively slower (weeks to months) colour change is achieved through pigment deposition. There is high energetic expenditure associated with deposition of melanin pigments and presumably other pigments, particularly those sequestered from the diet (Talloen *et al.*, 2004; Duarte *et al.*, 2017). Therefore, animals that have changed colour may choose backgrounds that prevent the high costs of further colour change. Choosing backgrounds may also incur lost foraging opportunities, or movement costs (Ruxton *et al.*, 2004), and so the combination of colour change and behavioural background choice may result as a trade-off of these costs (Polo-Cavia & Gomez-Mestre, 2017). We also know that matching in *B. betularia* is not perfect and that larvae cannot physiologically match very bright colours (chapter 2), and may therefore avoid resting on colours that they cannot match, as found in coral reef flounders (Tyrie *et al.*, 2015).

We found that brown *B. betularia* larvae were more likely to choose matching backgrounds over green larvae, particularly for the isoluminant treatment. This behaviour may relate to the ability of larvae to match the colour of dowels that they were reared upon, as brown *B. betularia* larvae were able to match brown dowels more closely than green larvae were able to match green dowels (chapter 2). Another reason may be because of differences in metabolic cost of melanin production *vs.* melanin degradation (Sugumaran, 2002; Mammone *et al.*, 2004). Perhaps it is more costly to remove pigmentation that turns larvae brown, than for a green individual to melanise, and so brown larvae choose to rest on backgrounds that minimise colour change, therefore metabolic costs.

In larval newts, unpigmented morphs choose paler backgrounds, whereas pigmented newts had no background preference (Polo-Cavia & Gomez-Mestre, 2017), and the same was reported in colour-changing juvenile flatfish, where pale phenotypes preferred pale sediment and darker phenotypes showed no colour preference (Ryer *et al.*, 2008). Here it was suggested that the lack of preference observed for dark morphs was because of an innate preference for light substrates, competing with background matching behaviour to create a neutral response (Ryer *et al.*, 2008). Our experiment would benefit from reciprocal mismatch trials to detect any innate preference and to find out if matching behaviour arises from a preference for a colour that larvae have been exposed to prior to the study, or from a physiological feedback loop driven by their own body colour (e.g., rear larvae on brown dowels until they are brown, then expose them to green dowels prior to choice trials).

Our results suggest that larvae are able to discriminate between luminance and chroma more readily than chroma alone. This effect has been found in other species; for example, frogs respond to luminance cues more readily than chroma cues to match backgrounds (Stevens *et al.*, 2014a; Stevens *et al.*, 2014b; Polo-Cavia *et al.*, 2016). Most studies on behavioural background choice only test luminance, offering a choice of light or dark backgrounds, or do not separate hue from luminance (Garcia & Sih, 2003; Polo-Cavia & Gomez-Mestre, 2017). However, this effect could be because larvae that had been reared on the extreme luminance dowels were slightly higher in greenness on average, compared to larvae from isoluminant dowels (chapter 2). Rearing larvae on isoluminant dowels and placing them in choice chambers containing extreme dowels would test this theory.

The most surprising finding of these experiments was that blindfolded larvae were able to choose habitats that are expected to reduce their detection, as effectively as control, non-blindfolded, larvae. As other cues, including diet, texture and temperature, were controlled for, we must conclude that larvae are processing visual information from their environment using both the ocelli and photoreceptors outside the eye, or extraocular photoreceptors (EOPs). Thus, not only are these larvae able to use EOPs as part of a mechanism to change their external appearance (chapter 3), but they can also use this EOP machinery to elicit a behavioural response. In principle, whereas colour change could be achieved by means of a local mechanism in the dermis, this latter finding strongly suggests the involvement of the central nervous system to integrate information and determine a whole body response.

EOPs that decrease detection through behavioural light avoidance have been reported in a species of pond snail (*Lymnaea stagnalis*) and *Drosophila melanogaster* larvae (Chono *et al.*, 2002; Xiang *et al.*, 2010), but this is the first example of behavioural background matching mediated by EOPs. However, exploration of EOP function is scarce, especially for behavioural background matching. A study on grasshoppers (*Acrida conica* Fabricius) found partial evidence for non-random background selection in blindfolded individuals, suggesting that the cues were not ocular, but did not explore the possibility of EOPs (Calver & Bradley, 1991). Cuttlefish (*Sepia officianalis*) change colour to match backgrounds (Buresch *et al.*, 2011), position themselves to increase crypsis (Mathger *et al.*, 2010; Barbosa *et al.*, 2012), and possess dermal EOPs (Kingston *et al.*, 2015). There is no direct evidence to suggest that cuttlefish use EOPs to adjust their position for reduced detection, but this coupled with more investigation into cephalopod body positioning in the context of camouflage, could be a subject for future work.

As with *Drosophila* larvae and other arthropod larval stages, *B. betularia* larvae have primitive eyes (ocelli) located in the head (Gilbert, 1994). Light sensing outside of the eye would allow larvae to sense their background from segments of the body that are directly resting on those backgrounds, which may be useful in a visually heterogeneous environment. We do not know the exact mechanism for how *B. betularia* larvae use EOPs for behavioural background matching, but it is possible that sensory neurons located in dermal tissue could be providing colour information to the central nervous system via a feedback loop, inducing avoidance/preference for particular colours. A similar mechanism is described in *D. melanogaster* where firing

of neurons that tile the body wall induced an escape response from light (Xiang *et al.*, 2010). This is the first evidence of behavioural colour matching in lepidopteran larvae, but early thorn (*Selenia dentaria*) caterpillars choose size-matched twigs to increase crypsis when masquerading as twigs. Although, it is uncertain as to whether visual cues are used for this behaviour (Skelhorn & Ruxton, 2012).

**CONCLUSIONS**

Our results appear to be the first example of EOPs mediating behavioural background choice. The adult moths of *B. betularia* are polymorphic for melanism, with each individual displaying a fixed phenotype. To reduce detection from avian predators, they too have been found to choose matching backgrounds (Kettlewell, 1955; Kettlewell & Conn, 1977). The larval stages of *B. betularia* are able to change colour to match a variety of visual environments they may find themselves in, but because this can take weeks to complete, individuals may choose to rest on backgrounds that match their colour to avoid predation during this time. This behavioural adaptation is thought to increase visual concealment from avian predators. However, we have not empirically tested whether birds find it more difficult to detect larvae on matching backgrounds, as opposed to mismatching backgrounds, and this could be considered for future work. It would also be useful to measure the metabolic cost of colour change and behavioural habitat choice in *B. betularia* larvae, to aid our understanding of why different camouflage strategies are employed and when they are most beneficial, both in terms of cost and predator avoidance.

# Chapter 5

## Mechanism of photoreception in *Biston betularia*: visual genes and electrophysiology


### ABSTRACT

Phototransduction is a well-characterised sensory pathway in animals and has been linked to numerous behavioural and physiological responses. An example of this is colour change, where animals use visual information to improve their match to the colour and pattern of their surroundings. In some cases, visual camouflage is achieved using light sensing outside of the eye, with cells known as extraocular photoreceptors (EOPs). The molecular basis of EOPs in chromatophores has been explored, but little is known about EOPs that contribute to slow, morphological colour change. Peppered moth (*Biston betularia*) larvae change colour to match the twigs of their host plants, without the use of their eyes. We measured expression of colour-detecting opsin genes, and other key visual genes, in the eye and across the whole body dermis of larval and adult stages of *B. betularia*. We also looked for evidence of phototransduction in response to lights of varied wavelengths. In larvae, we found high dermal expression of visual genes relative to eye expression and electrochemical signals in response to UV, blue, green, and amber light. These results provide further evidence for dermal photoreception in *B. betularia* larvae and increase our knowledge of the molecular basis of vision in this species. Studying the molecular and physiological basis of vision is important to provide a complete understanding of colour change, and other visually-induced behaviours in animals.


### INTRODUCTION

Animal sensory systems can provide useful insight into the origins and evolution of complex physiological and behavioural traits. One of the best-understood sensory pathways in animals is photosensitivity, where opsin-mediated phototransduction cascades convert light photons to electrical signals (Hardie, 2001; Fu & Yau, 2007). These molecular pathways have been described predominantly for retinal phototransduction, but there is a growing body of information on photoreception outside of the retina, used for a variety of purposes, including light avoidance (Pankey *et al.*, 2010; Xiang *et al.*, 2010), mating (Arikawa *et al.*, 1997), and colour change (Buresch *et al.*, 2011; Fulgione *et al.*, 2014). Human skin contains visual gene transcripts, thought to trigger melanin production, increasing protection from potentially harmful UV light (Haltaufderhyde *et al.*, 2015; Toh *et al.*, 2016).

Some animals use visual cues from their environment to change the colour and/or pattern of their body to match their surroundings, thus reducing detection by visual

hunters (Stevens *et al.*, 2014b; Hultgren & Mittelstaedt, 2015). This response requires the animal to perceive colour and, in some cases, detailed patterns; but the underlying visual cascade has not been explicitly examined or linked to colour change, for example in several fish and arthropod studies (Grayson & Edmunds, 1989; Kelman *et al.*, 2006; Stevens *et al.*, 2014b; Stevens *et al.*, 2015). In some colour change studies, light-sensitive opsins have been characterised, but this is predominantly in animals displaying fast physiological colour change such as amphibians and cephalopods (Messenger, 2001; Choi & Jang, 2014). In these animals, opsins located in chromatophores provide a localised neural network, where visual information elicits chromatophore contraction followed by colour change (Oshima, 2001). This localised response has been demonstrated in isolated dermal tissue from numerous lizard species, which are able to detect light and subsequently change colour (Hadley, 1928; Smith, 1929).

Isolated melanophores from tadpoles of the African clawed frog, *Xenopus laevis*, were found to disperse in response to illumination (Daniolos *et al.*, 1990), using melanopsin, a non-visual class of opsin (Provencio *et al.*, 1998). Adult frogs also use chromatophores to change colour in response to visual backgrounds (Kang *et al.*, 2016), but the diversity of wavelength specific opsins expressed in these chromatophores was not explored (Okano *et al.*, 2000). Unlike amphibians and reptiles, which express melanopsin in dermal melanophores, fish chromatophores express a wide variety of cone opsins, which respond to wavelengths ranging from 380-580 nm (Chen *et al.*, 2013). Cuttlefish and squid chromatophores contain identical opsin transcripts as those found in their retinas (Kingston *et al.*, 2015; Ramirez & Oakley, 2015). In cuttlefish, opsin expression appears to be localised to the ventral mantle and dorsal fin, in addition to the retina, but expression was absent in other dermal tissues (Mathger *et al.*, 2010).

Despite the existence of localised light sensitivity in the chromatophores of numerous species, the eyes still play an important role in some colour change systems. For example, the melanophores in Fiddler crabs, *Uca pugilator*, exhibit two responses: the primary response is a localised dermal response to illumination, causing the animal to darken in colour (Brown & Sandeen, 1948); the secondary response involves the eyes and allows the animals to match the luminance of their substrate (Rao & Fingerman, 1968). Colour change in Dungeness crabs, *Cancer*

*magister*, is dependent on the eyes; when the eyestalks were removed, melanophores were unable to disperse, resulting in blanching of the crab dermis (Shibley, 1968).

Compared with rapid colour change, there is generally less information on slow colour change (see Introduction, chapter 2). Vision has been linked with slow colour change in studies on crabs and hawkmoth larvae (Grayson & Edmunds, 1989; Stevens *et al.*, 2014b), but there has been very little exploration of the molecular basis of vision, both in the retina and putative EOPs. Crab spiders, *Misumena vatia*, change colour in response to visual stimuli through the formation of ommochrome granules (Insausti & Casas, 2008). Electrophysiological and morphological evidence suggests that *M. vatia* can perceive colours from UV to orange (Defrize *et al.*, 2011), and their visual field can simultaneously analyse their own colour and the colour of the substrate (Insausti *et al.*, 2012).

Larvae of the peppered moth (*Biston betularia*) use visual cues to change colour to match the twigs upon which they rest (Noor *et al*., 2008; chapter 2). My own experiments (chapter 3) indicate that larvae use EOPs to sense their visual surroundings preceding colour change, but the molecular and physiological basis of vision in this species had not been studied. To help us better understand colour change in *B. betularia* we addressed the following questions:

1. Are *B. betularia* capable of colour vision in both the larval ocelli and the adult compound eye?
2. Are putative EOPs in *B. betularia* larvae capable of colour vision?

As opsins are essential molecules for colour vision (Shichida & Matsuyama, 2009), we first characterised visual (UV, blue, LW1, LW2), and non-visual (melanopsin) opsin gene sequences from partial whole genome sequence of *B. betularia* (see Table S5.1 for gene information). We measured expression of these genes in retinal and dermal tissues of adult moths and larvae to determine the range of colours that are perceived in the eye. Dermal opsin expression would provide further evidence for EOPs in larvae. However, opsin expression alone is considered weak evidence for extraocular vision (Ramirez & Oakley, 2015). Therefore, we identified and measured expression of two additional visual genes: arrestin-1 (arr), which plays a critical role in quenching phototransduction by binding opsin (Hargrave, 2001) and retinal degeneration-B (RDB), which codes for a protein required for prevention of photoreceptor degeneration (Harris & Stark, 1977). To determine physiological

responses to light and colour in *B. betularia*, we presented larvae with wavelengths of light corresponding to expressed opsins and recorded electrical signals as evidence for phototransduction. Extracellular recordings were taken from the eyes (ocelli) and dermal tissue of individuals.

## METHODS

### Opsin identification

An incomplete *Biston betularia* whole genome assembly (Roche 454) was already available (Saccheri lab, University of Liverpool), along with a pooled whole genome BAC library, assembled by Amplicon Express. Predicted sequences for opsin genes were gained by aligning contiguous sequence reads from the *Biston betularia* WGS by tBLASTn (Altschul *et al.*, 1997) with homologous *Manduca sexta* sequence, using Geneious v.5.5.6 (Kearse *et al*., 2012).

Primer pairs (Table S5.3) were designed from predicted sequences, for detection and amplification of opsin genes in the BAC library. Round one PCR was performed on eight superpools (2,688 BAC clones from seven plates + one positive control, containing all BAC clones in the library). Any positive hits were pursued by a round two PCR on the corresponding matrix pool for that plate. Reading positive hits allowed identification of the plate and exact well position of each clone of interest. PCR reactions were performed using 2,500 U/mL LongAmp® Hot Start Taq DNA Polymerase (New England Biolabs) in cycling reactions as follows: 2 min at 94$^{o}$C, 40 cycles of [20 s at 94$^{o}$C, 30 s at 57$^{o}$C, 1 min at 70$^{o}$C], in an Applied Biosystems Veriti 96 well thermal cycler. PCR products were loaded onto a 2% agarose gel and visualised with Ethidium bromide (Sigma-Aldrich) against Hyperladder 50bp (Bioline).

Clones of interest were grown in 2 mL of LB (luria broth) medium and DNA was isolated using a FosmidMAX™ DNA Purification Kit (Epicentre Biotechnologies). Clones were cycle sequenced using Bigdye terminator v3.1 (Applied Biosystems), with gene-specific forward and reverse primers, plus standard end forward and reverse sequencing primers, T7 and FosmR (Table S5.3), under cycling conditions of: 1 min at 96$^{o}$C, 150 cycles of [30 s at 96$^{o}$C, 12 s at 50$^{o}$C and 4 min at 60$^{o}$C]. Products from cycle sequencing were suspended using HiDi Formamide (Applied Biosystems), and resolved with an Applied Biosystems Hitatchi 3130 *xl* genetic analyser.

To ensure that *B. betularia* visual genes were true orthologues, sequences (Fig. S5.1) were aligned with corresponding genes of closely related Lepidoptera species (Table S5.4), obtained using a combination of NCBI BLAST. Sequences were aligned manually in MEGA6 v.6.0 (Tamura *et al.*, 2013) and model selection was performed on nucleotide substitutions using the Maximum Likelihood statistical method for all sites, with complete deletion of gaps/missing data.

Phylogenetic trees for each gene were then constructed from nucleotide substitutions using Maximum Likelihood. The model used was dependent on results from model selection, from which we selected the best-fitting model using AICc and BIC values. For UV opsin nucleotide sequences, this was the Tamura 3-parameter model with a discrete Gamma distribution used to measure evolutionary differences among sites. For blue opsin and arrestin-1 nucleotide sequences, the Tamura 3-parameter model was also used, with a discrete Gamma distribution and 5 rate categories, assuming that a certain fraction of sites are evolutionarily invariable. For LW sequences, the General Time Reversible model was used, with a discrete Gamma distribution and 5 rate categories, assuming that a certain fraction of sites are evolutionarily invariable. For RDB sequences, the General Time Reversible model was used, with a discrete Gamma distribution. Each phylogeny was constructed using all codon positions and analysis was run using 2000 bootstrap replications. Trees were constructed in MEGA6 v.6.0 (Tamura *et al.*, 2013) and edited in Figtree v1.4.3.

**Visual gene expression**

*Biston betularia* larvae were reared on a constant supply of grey willow (Salix cinerea) until final instar, when individuals were euthanised by flash freezing at -80$^{o}$C. Individuals were then immediately thawed and the gut of each was removed and discarded, and the remaining tissue was placed in RNAlater (Thermofisher) until required. Four larvae were chosen at random from a sibling group (Table S5.2), and dissected into four tissue parts: head, thorax, abdomen, and claspers (Fig. 5.1A). Four adults (two male, two female) from separate crosses (Table S5.2) were euthanised in the same way as larvae, two days after eclosion. Adults were dissected into four tissue parts: head, thorax, abdomen, and genitalia (Fig. 5.1B). The adult stage of *B. betularia* does not feed and so does not possess a gut; however, as much internal tissue as possible was removed from the thorax, abdomen, and genitalia, leaving only dermal tissue.

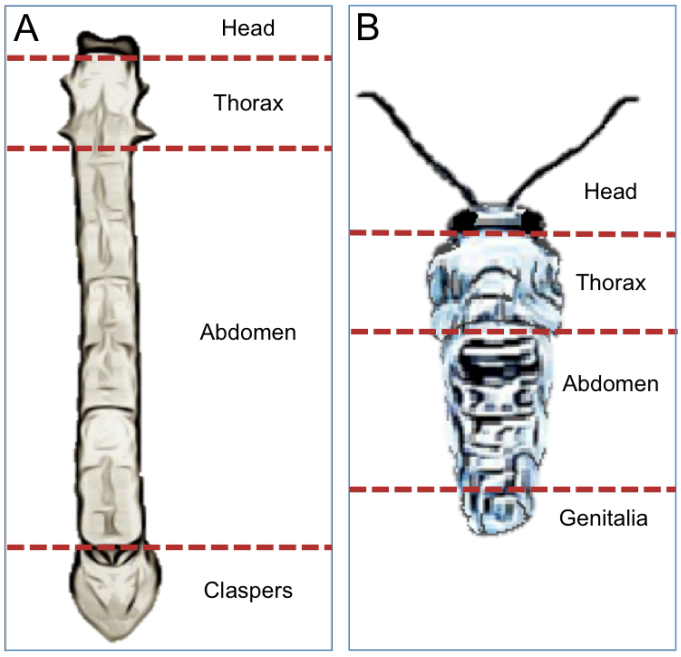


**Figure 5.1. Dissection of tissues in *B. betularia*.** (A) Four tissue parts of larvae: head, thorax, abdomen, and claspers. (B) Four tissue sections of adults: head, thorax, abdomen, and gentitalia. Red dashed line indicates where tissues were separated during dissection.

For RNA extraction, all tissue was placed in clean 1.5 mL Eppendorf Safe-Lock Tubes, each containing a 3 mm tungsten bead (Qiagen), and 1 mL of TRIzol reagent (Thermofisher) was added. Samples were homogenised with a Qiagen Tissue Lyser II, at 25 Hz for 4 minutes. Total RNA was isolated following the recommended procedure. Genomic DNA was removed from 6 µL of each RNA sample by DNase I, Amplification Grade (1 U/µL; Thermofisher), following the manufacturer's protocol. First strand cDNA was synthesised from 5 µL of DNase-treated RNA using 200 U/µL Superscript III Reverse Transcriptase (Thermofisher), following a modified version of the recommended protocol, excluding the RNaseOUT stage and using 0.5 µL of 100 µM Oligo (dT20) instead of 1 µL of 50 µM Oligo (dT20) as the anchor primer. Reactions were incubated at 50$^{o}$C for 60 minutes, followed by deactivation at 70$^{o}$C for 15 minutes.

Primers for qPCR (Table S5.2) were designed in OLIGO (v.6.0) to test the expression levels of 7 opsin genes: ultraviolet (UV), blue splice variant 1 (BS1), blue splice variant 2 (BS2), long wavelength copies 1 (LW1), and 2 (LW2), melanopsin splice variants A (melA), and B (melB). We also tested expression levels of two other visual genes: arrestin-1 (arr) and retinal degeneration B (RDB). Gene expression for all visual genes was measured against two uniformly expressed control genes (spectrin and Rps3A). As both of these control genes provided the same results, all analyses were conducted using spectrin only. Preceding qPCR,

primers were optimised for annealing temperature by running end-point PCR temperature gradients on an Applied Biosystems Veriti 96 well thermal cycler.

Quantitative PCR (qPCR) was performed using KAPA SYBR fast qPCR (2X) mastermix (KAPA Biosystems), following the manufacturer's protocol to provide a reaction mixture of 0.5 µL cDNA template (diluted to 55% in nuclease-free water), in a final reaction volume of 10 µL. Each sample was repeated in triplicate and quantified using a Roche 480 ii lightcycler, under cycling conditions: [3 min at 95$^{o}$C, 45 cycles of 3 s at 95$^{o}$C, 20 s at optimal annealing temperature, 20 s at 72$^{o}$C]. Melting curve analysis was calculated to ensure single products. Both this, and relative quantification were calculated using the Roche 480 Lightcycler software (v.1.5). Relative expression of PCR product was determined as a ratio using [(ERef)^(CpRef)] / [(ETarget)^(CpTarget)], where E=efficiency of PCR reaction, and Cp= crossing point.

**Electrophysiology**

All electrophysiology experiments were performed in Mikko Juusola's lab, University of Sheffield. Light stimuli were provided by high- intensity light-emitting diodes (Marl Optosource), driven by a custom-built LED driver. Microelectrodes were pulled with a horizontal laser puller (P-2000; Sutter Instrument Company) from glass capillaries, with an inner and outer diameter of 0.5 and 1.0 mm, respectively. Electrodes were back-filled with fly Ringer's solution, chloridised silver wires were inserted, and these were mounted onto a manual micromanipulator. Recordings were taken at 20$^{o}$C (room temperature, controlled by air conditioning). Each individual was dark-adapted for five minutes before each recording was performed in a dark room behind a black-out curtain. The stimulus generation, data acquisition, and signal analysis was performed by a custom-written program based on the MATLAB programming language Mathworks (BIOSYST, M. Juusola, 1997-2008).

***Eye/ocelli recordings***

Live *B. betularia* larvae were cooled by brief refrigeration for 5 minutes, to reduce movement, and then fixed into a cone-shaped holder (a hollow copper core surrounded by a ceramic insulator) using beeswax, so that only the head was exposed (Fig. 5.2A). A reference electrode was placed on the top of the head, making light contact with the epidermis. A recording electrode was placed on the lateral side, making light contact with all six ocelli (bridged by Ringer's solution). Larvae were exposed to one-second pulses of 4V light stimuli and responses to four wavelengths of light were recorded: amber (590 nm), green (530 nm), blue (420 nm), ultraviolet (350 nm). Each wavelength is the average ±20 nm.

To reduce any effect of previous exposure, the order of different wavelengths that larvae were exposed to was randomised. A Cardan arm system allowed free movement of the light source around the specimen, keeping a constant distance of 50 mm from the surface of the ocelli.

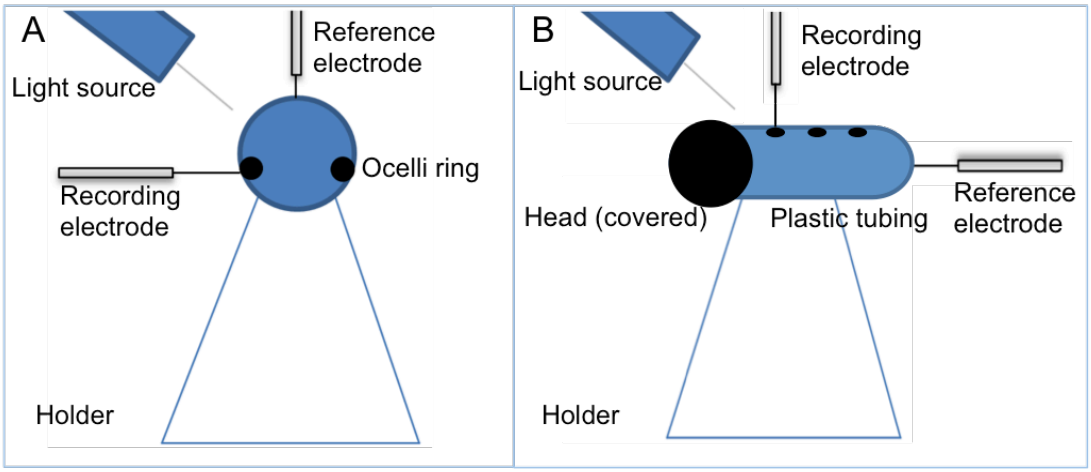


**Figure 5.2. Schematic of set up for *in vivo* extracellular recordings of *B. betularia* in response to chromatic light.** (A) Set up for eye/ocelli recordings; (B) set up for dermal recordings in live larvae.

***Dermal recordings***

Live *B. betularia* larvae were cooled by brief refrigeration (5 minutes), to reduce movement, then fixed into plastic tubing with a series of small holes (3 mm diameter) in the top using beeswax so that only the head was exposed (Figure 5.2B). The plastic tubing was placed on the same ceramic insulator used for eye recordings. The entire head of the larva was painted with black acrylic paint and covered with a small foil shield to stop light penetrating the ocelli. A reference electrode was placed on the posterior end of the larvae, making light contact with the epidermis. A recording electrode was placed into the holes in the tubing, making light contact with

various parts of the epidermis. Larvae were exposed to 4V light pulses ranging from 1-10 seconds, and responses to the same four wavelengths of light were recorded, as described for ocelli recordings.

Dermal recordings were also taken on isolated dermal tissue. The head and gut was removed from larvae and the skin was pulled back from a ventral incision and fixed with pins into a plastic petri dish (60mm diameter). This was then attached to the ceramic insulator. A reference electrode was placed onto the top of the thorax making light contact with the epidermis. A recording electrode was placed at different position along the thorax, abdomen, and claspers. Dermal tissue was exposed to a 1-10 second 4V light pulse and responses were recorded to the same four wavelengths of light described for previous tissues with the addition of white light.

**Statistical analysis**

***Visual gene expression***

To test whether dermal skin expression was higher in larvae compared to adults, we first combined relative expression from all tissue parts including the head to give total expression values for each individual. We then calculated dermal expression (thorax, abdomen, claspers/genitalia) as a proportion of total expression. We modelled dermal expression using beta regression, as it was proportional data and followed a beta distribution. We tested stage as predictors of dermal expression and model residuals were checked using qqPlot in R version 3.3.2 (R Core Team, 2014).

***Electrophysiology***

To test the response of larval ocelli to different wavelengths of light, values for receptor potential were extracted from extracellular recordings (Fig. 5.7). We tested colour and individual as predictors of receptor potential, using a two-way ANOVA. We checked normality of data and model residuals using qqPlot. All statistics were performed using R version 3.3.2 (R Core Team, 2014).

**RESULTS**

**Opsin identification**

A total of six opsin gene copies, not including UV and blue splice variants, were identified in the *B. betularia* genome and sequenced from tissue: Ultraviolet (UV); blue; two copies of long wavelength, LW1 and LW2; and two copies of melanopsin, melA and melB. Two additional visual genes, arrestin-1 (arr) and retinal

degeneration B (RDB) were also identified, (see Fig. S5.1 for sequences). All visual genes were homologous with visual genes of closely related Lepidoptera and tree topology showed *Biston betularia* as sister taxa to other species of Geometridae, for genes where the molecular sequences for geometrids were available (Fig. 5.3). LW1 in *B. betularia* showed a closer homology to LW1 genes in other geometrids, than to the second copy of LW2 in *B. betularia*.

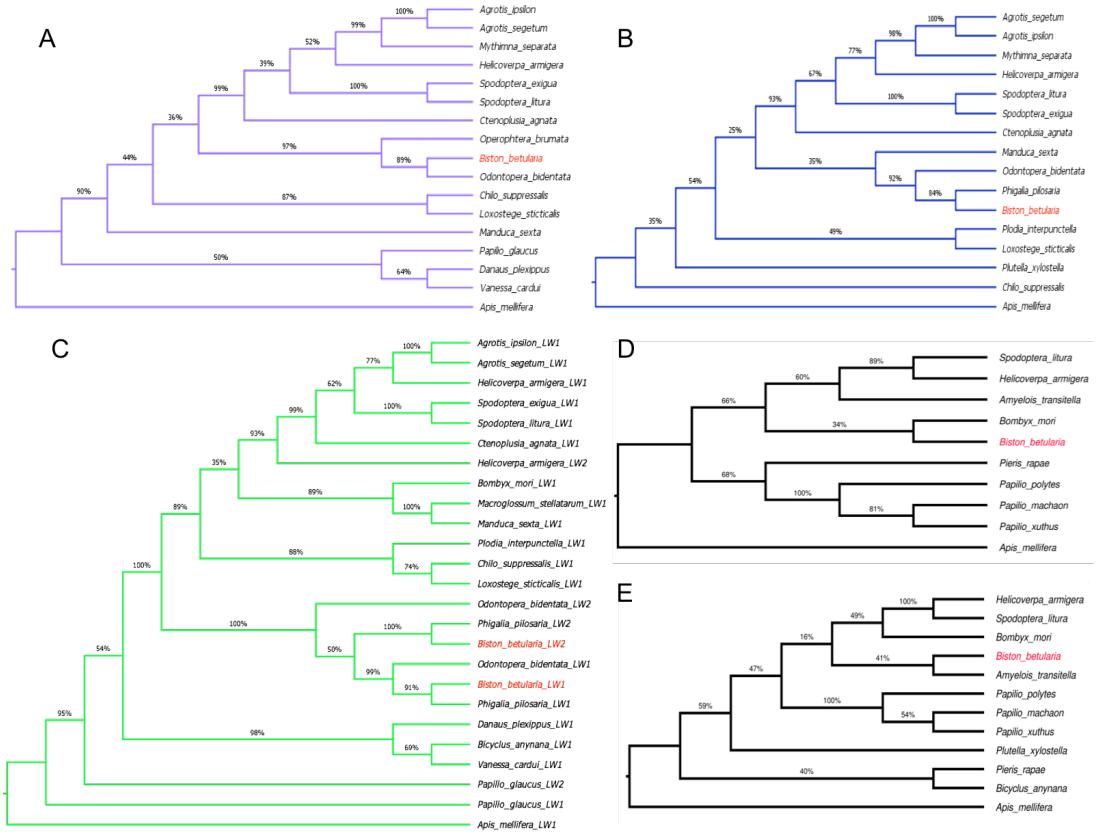


**Figure 5.3. Phylogenetic position of *B. betularia*** (shown in red) visual genes with respect to homologous genes from a sample of other Lepidoptera (Table S5.4) as inferred from ML analysis. A= ultraviolet-sensitive opsin (UV), B= blue-sensitive opsin (Bl), C= Long wavelength-sensitive opsins copies 1 and 2 (LW1, LW2), D= arrestin-1 (arr), E= retinal degeneration B (RDB). Bootstrap percentages based on 2000 replications are displayed on corresponding branches. Cladogram rooted on *Apis mellifera*, evolutionary analyses were conducted in MEGA6 v.6.0 (Tamura *et al*., 2013).

## Visual gene expression

Blue and UV opsin showed alternative splicing, producing variants UV2 and BS2 in larval and adult *B. betularia* tissues. Two splice variants of melanopsin were also detected (Fig. 5.4). UV2 was excluded from qPCR analysis due to amplification difficulty.

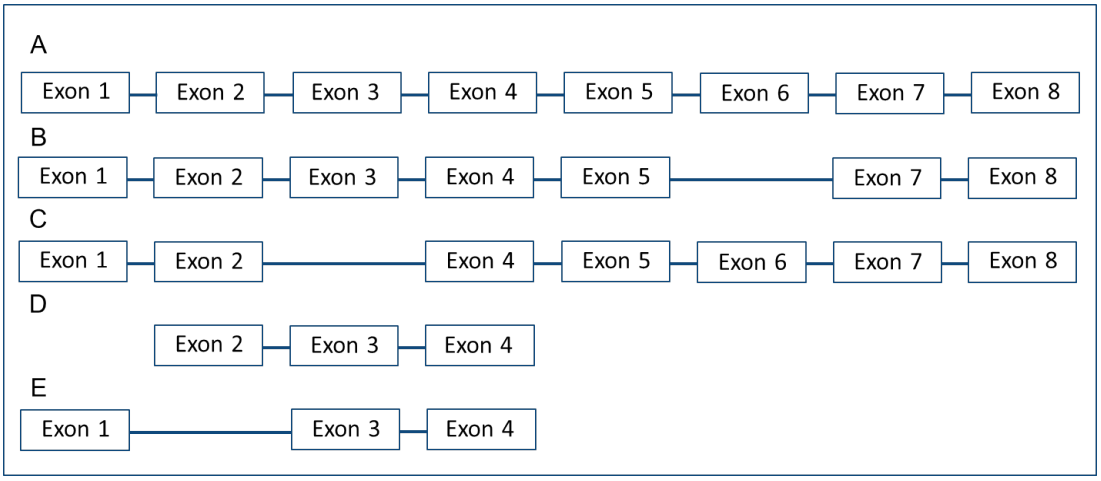


**Figure 5.4. Exon structure of opsin genes including splice variants.** (A) full gene copy in UV, BS1, LW1, and LW2. (B) BS2, lacking exon 6. (C) UV2, lacking exon 3. (D) melanopsin A, in which exon 2 acts as the first exon. (E) melanopsin B, with exon 2 skipped.

Larval and adult stages of *B. betularia* expressed all visual genes to some extent, in both head and dermal tissues (Figs. 5.5a, 5.5b). Generally, dermal expression in larvae was significantly higher, in relation to head expression, than in adults (Fig 5.6; $Z_{11}$=0.22, $P$<0.0001), where RDB ($Z_{11}$=0.44, $P$<0.0001), BS2 ($Z_{11}$=0.45, $P$<0.001), melA and melB ($Z_{11}$=0.44, $P$<0.0001) showed significantly higher dermal expression compared with other genes. In larvae, arrestin was only 10 times higher in the head than dermal tissue and LW1, RDB, and melB expression was uniform across head and body tissue (Fig. 5.5a). For RDB and melB, expression was higher in larval dermal tissues than in head tissue, with 70% of melB and 60% of RDB expressed in the skin (Fig. 5.6). BS2 was also highly expressed in larvae dermal tissue, with around 50% in the dermis and 50% expressed in the head (Fig. 5.6).

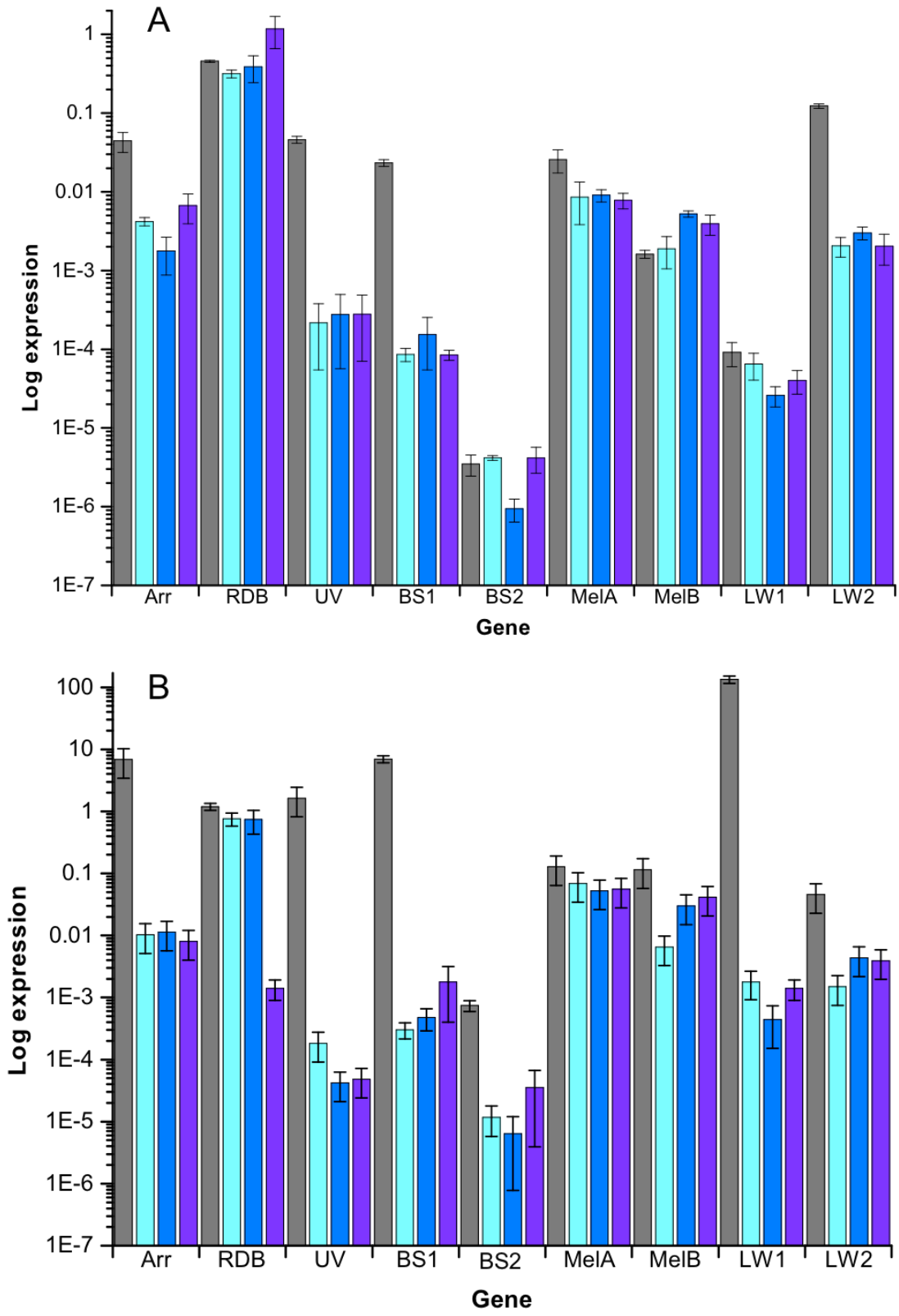


**Figure 5.5. Visual gene expression in *B. betularia* larvae.** Log mean (± SE) expression of visual genes, relative to reference gene spectrin, in *B. betularia* larval (A), and adult (B) head (grey) and dermal tissue of thorax (cyan), abdomen (blue), and claspers in larvae/genitalia in adult (purple). *n*=4 for each bar.

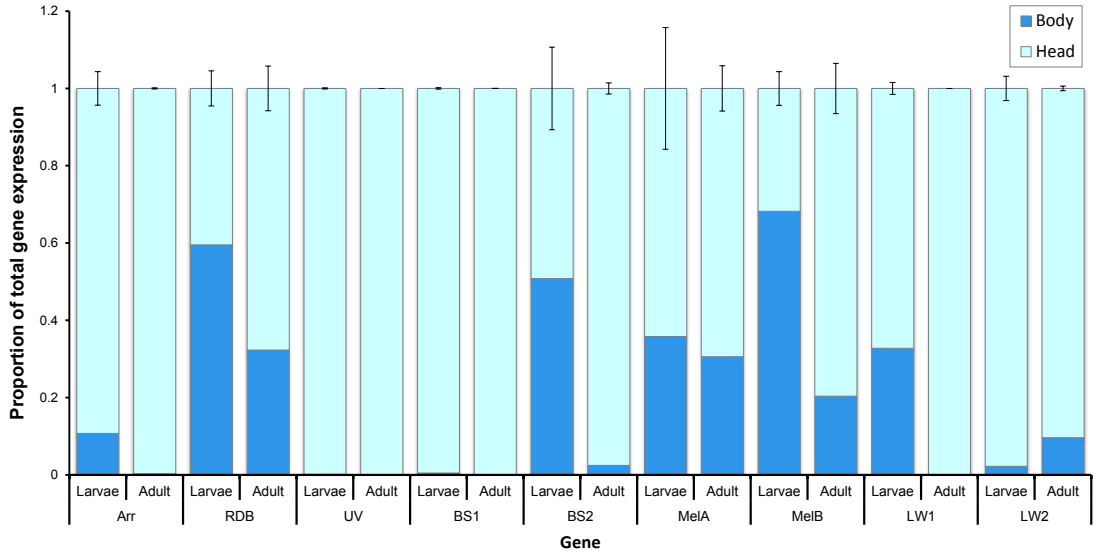


**Figure 5.6. Visual gene expression in head and dermal (body) tissues.** Expression as a proportion of total expression in larval and adult stages of *B. betularia*. Ratios calculated from average expression levels shown in Figs. 5.5a, 5.5b. Error bars show standard error from 4 individuals of each stage.

By contrast, in adults, head expression constituted nearly 100% of total visual genes expression, with the exception of both melanopsin variants and RDB, with ~30% dermal expression for these genes (Fig. 5.6). In adults, arrestin, UV, BS1, and LW1 was approximately 1,000 times higher than dermal expression (Fig. 5.5b).

Dermal: head expression for LW1 and LW2 differed between larval and adult life stages, in opposing directions. LW1 is relatively up-regulated (35%) in larval dermis, and down-regulated in adult dermis to near zero (Fig. 5.6). LW2 shows an opposing pattern, with a higher proportion of dermal expression in adults (10%), compared with 3% in larvae (Fig 5.6).

**Electrophysiology**

Photoreceptors in the ocelli of *B. betularia* larvae responded to all four wavelengths of light we tested (Fig. 5.7). The receptor potential in ocelli appeared to decrease slightly with increasing wavelength, where it was highest for UV and lowest for amber light (Fig 5.8). However, these differences were not significant ($F_3$=0.67, $P$=0.59; Fig. 5.8A). The large variance in response to wavelength was due to large differences in response between individuals ($F_3$=6.03, $P$=0.016; Fig 5.8B), where individual one showed a higher response across all wavelengths ($P$=0.013). We obtained recordings of dermal responses in one larva (Fig. 5.9), which showed a much slower response in comparison to ocelli recordings.

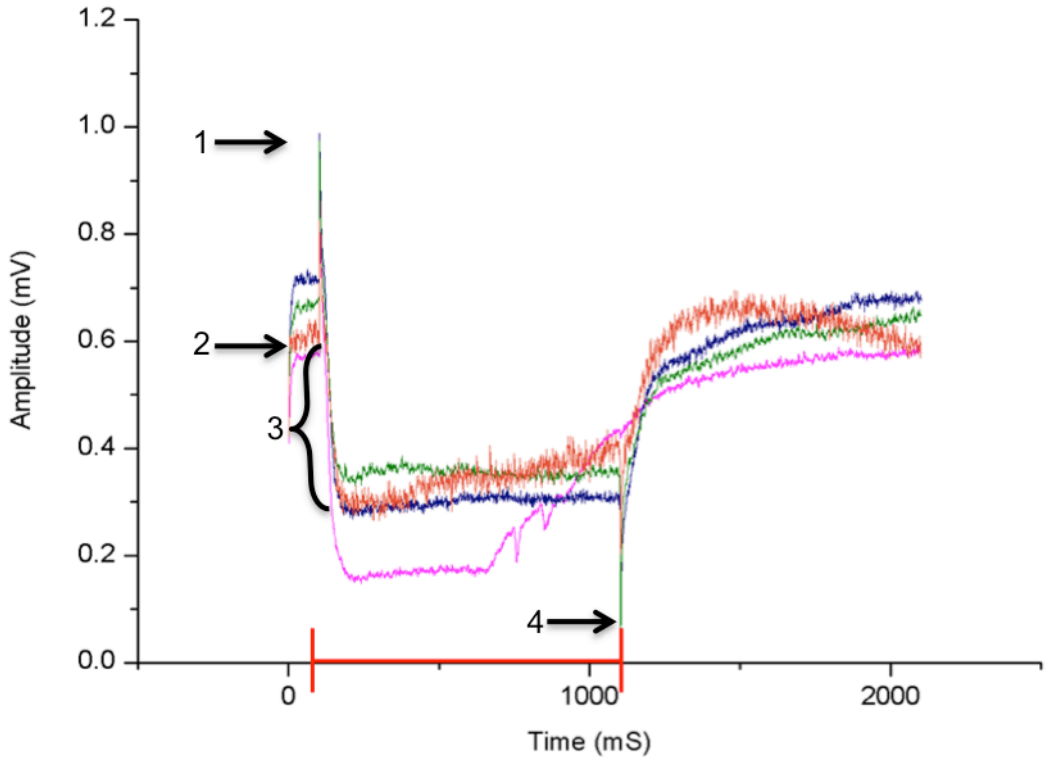


**Figure 5.7. Extracellular recordings from *B. betularia* larval ocelli.** Electroretinogram signals recorded from *B. betularia* ocelli in response to 1-second pulses of UV (magenta), blue (blue), green (green), and amber (orange) light. Light pulse shown by red line on x-axis, with caps showing on and off time points. Recordings per individual, *n*= 10. Individual, *n*=5. 1= 'on' transient potential, 2= baseline (zero) potential, 3= receptor potential amplitude, 4= 'off' transient potential.

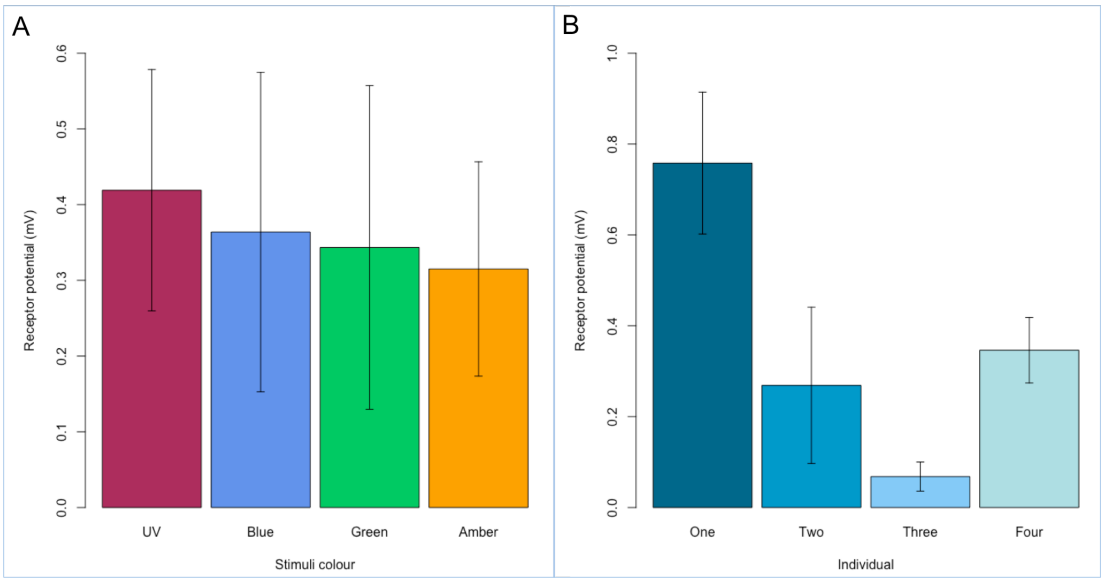


**Figure 5.8. Evoked response (receptor potential) of ocelli photoreceptors to 1-second light pulses of four different wavelengths.** (A) Comparison of responses to different wavelengths, averaged across individuals (*n*=4). (B) Comparison of responses in individual larvae, averaged across light wavelengths (*n*=4).

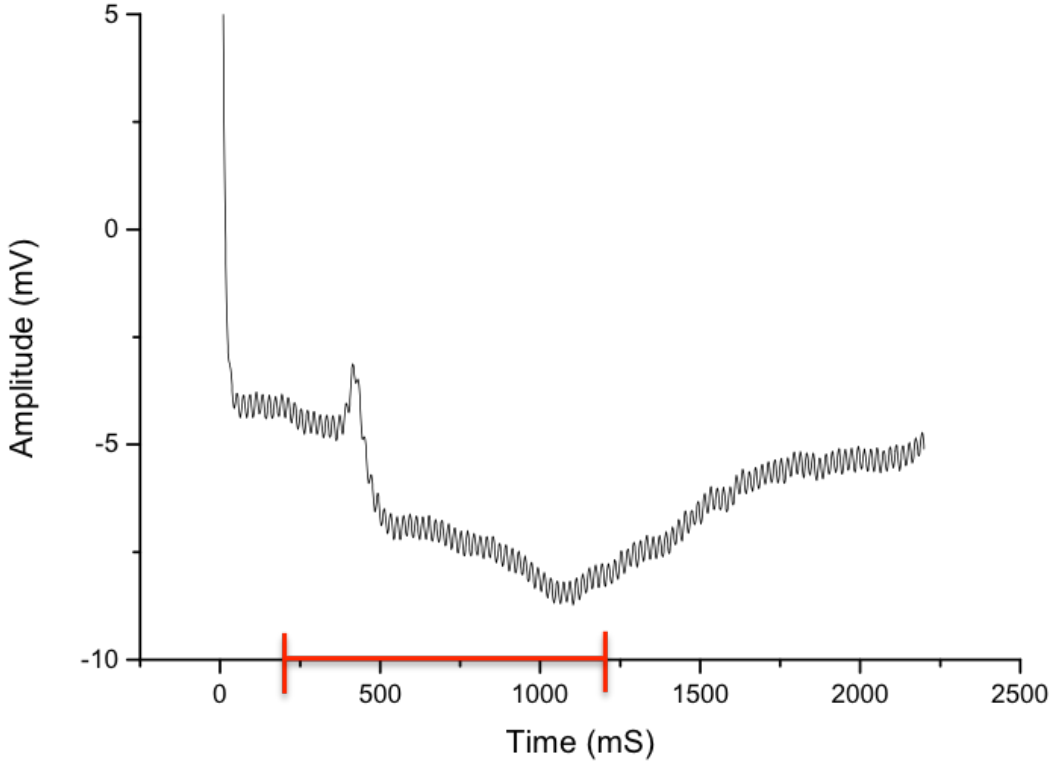


**Figure 5.9. Extracellular recordings from *B. betularia* larval dermis.** Electroretinogram signals recorded from isolated *B. betularia* dermis in response to 1-second pulses of white light (black line). Light pulse shown by red line on x-axis, with caps showing on and off time points. Recordings per individual, *n*= 10. Individual, *n*=1.

## DISCUSSION

We have shown that larvae and adult *B. betularia* express a full array of visual genes, not only in the ocelli and compound eye, but also in the body dermis. This result corroborates the finding that caterpillars are able to change colour without the use of their ocelli (chapter 3). The opsin classes detected (UV, blue, long wavelength) should, theoretically, provide spectral sensitivity to wavelengths ranging from 300-700 nm (UV, blue, green and amber). Ocular expression of opsins in the adult stage has been described in several other species of Lepidoptera (Briscoe, 2008; Xu *et al.*, 2013), and electrophysiology has provided evidence for spectral sensitivity to this wavelength range in the compound eye of many moths and butterflies (Agee, 1973; Bennett *et al.*, 1997; Imafuku, 2013; Telles *et al.*, 2014).

There have been far fewer studies of ocular vision (and none on EOP) in lepidopteran larvae, but electroretinograms have shown ocular sensitivity to a range of colours in the silkworm (Ishikawa, 1969), swallowtail butterflies (Ichikawa & Tateda, 1980), and a pest species, *Trabala vishnou* (Lin *et al.*, 2002). As gene expression is costly (Glick, 1995; Rang *et al.*, 2003), it would be unlikely that *B. betularia* moths express a suite of visual genes in their dermis that are not functional.

Opsins and visual arrestin were also found to be co-expressed in non-retinal tissues of a species of freshwater, eyeless polyp, *Hydra magnipapillata,* which is thought to mediate cnidocyte release (Plachetzki *et al.*, 2010; Plachetzki *et al.*, 2012).

That adult *B. betularia* express visual genes in their dermis is perhaps surprising. The colour (melanism pattern) morphs of *B. betularia* adult moths are genetically fixed, so vision is not used for colour change, as in the larvae. Nor do the adult moths feed, so colour vision would not be necessary for plant identification, as found in some nectar-feeding moths and butterflies (Cepero *et al.*, 2015; Ohashi *et al.*, 2015). However, similarly to the larvae (chapter 4), adult peppered moths have been found to choose colour-matching backgrounds to reduce detection from avian predators (Kettlewell, 1955; Kettlewell & Conn, 1977). Grant and Howlett (1988), fitted either dark or light collars to *B. betularia* adults to test if self-inspection of their own body scales determined their choice between dark and light resting substrates. Dark morphs had a preference for dark backgrounds, regardless of collar colour, and so the authors concluded that preference was genetically determined. This does not exclude the possibility that EOPs are used for self-inspection and inspection of the background. Other species of Geometrid moths have been found to inspect their visual background to improve crypsis against it, and are more likely to do so if their current crypticity is low, also suggesting self-inspection (Kang *et al.*, 2012; Kang *et al.*, 2013). Vision in these moth species has not yet been explored, but may not be processed in the eyes alone. EOPs have been described in adult Lepidoptera, where genital photoreceptors are proposed to aid in mate choice and oviposition in swallowtail butterflies (Arikawa & Miyako-Shimazaki, 1996; Arikawa *et al.*, 1997), but this has not been explored in moths.

In the larval eye, LW2 was most highly expressed, followed by UV and BS1, which showed similar expression. This is not consistent with the ERG potentials recorded for each wavelength, where shorter wavelengths (UV and blue) elicited a slightly larger response than long wavelength (green and amber). However, these differences were very small and masked by high variance between individual responses. The wavelengths used for recording the ERGs were broad and could stimulate more than one opsin in *B. betularia*, which makes it difficult to compare our electrophysiological and molecular results. It would make sense that we recorded similar responses across the wavelength range, as different parts of the larval ocelli are likely to contain photoreceptors sensitive to different wavelengths. For example,

in *T. vishnou* larvae, ocelli 1-4 (dorsal side) contain UV, blue and green photoreceptors, whereas ocelli 5 and 6 contain only blue and green receptors, and so are not sensitive to UV (Lin *et al.*, 2002). Swallowtail butterfly larvae also show differential colour sensitivity across ocelli (Ichikawa & Tateda, 1980). As we measured extracellular recordings with contact across all ocelli, each photoreceptor would contribute to the signal obtained. Generally, extracellular recordings are only used to test for a positive or negative response and so we would need to take intracellular recordings for each ocellus, increasing light intensity for each wavelength range, and perhaps use narrower ranges to gain accurate spectral sensitivity data for *B. betularia*.

Dermal expression of RDB, LW1 and BS2 was high in larvae. Differential expression of visual genes across the dermis suggests that some of these genes may play a larger role in non-ocular photoreception. Some studies have detected visual genes across the dermis and linked visual machinery with colour change, but have not compared expression levels between genes, or distinguished between opsin classes (Fulgione *et al.*, 2014; Kingston *et al.*, 2015). In these examples, visual gene expression has been located in chromatophores (Fulgione *et al.*, 2014; Kingston *et al.*, 2015). Chromatophores are not likely to be responsible for colour change in *B. betularia* larvae (chapter 2), and to our knowledge, visual genes have not yet been located in the dermis of any other animals that display morphological colour change, i.e. not using chromatophores.

In larvae and adults, the two copies of LW, and both blue splice variants showed differential dermal expression relative to the head. The fact that LW2 shows closer homology to LW2 genes in other geometrid species, than it does to *B. betularia* LW1 suggests these two copies evolved independently of one another. Therefore the difference in expression pattern is not surprising. Opsin gene duplication has been intensively studied in Lepidoptera (Briscoe & Chittka, 2001; Briscoe, 2008), and it had been suggested that duplicate genes fall into one of two categories: subfunctionalisation or neofunctionalisation (Briscoe, 2008). In this context, subfunctionalisation describes a situation where one or both of the paralogues acquires a more restricted expression pattern/biological activity than the ancestral gene. Neofunctionalisation is where paralogues are free to acquire new functions/expression patterns (Force *et al.*, 1999). LW1 expression was high in comparison to LW2, and BS2 showed higher expression in the thorax and claspers of

larvae than in the head. It is possible that these gene variants may have arisen with the function of non-ocular light/colour detection (neofunctionalisation). For *B. betularia* larvae, high expression of LW genes may be expected, as the colours commonly seen in nature would be in the medium to longwave range of the spectrum, i.e. green and brown.

Some studies have found that opsin expression alters due to diet and light conditions (Xu *et al.*, 2013; Yan *et al.*, 2014). Environmental factors were controlled in our experiments, which kept variation low between individuals. However, it would be interesting to see if visual genes in *B. betularia* larvae are downregulated during decreasing light intensity, and if this pattern is the same in retinal and dermal opsins, as this may provide more evidence of the function of these genes.

We are currently unaware of the exact phototransduction pathway that occurs in response to light/colour in *B. betularia* dermal photoreceptors. It has previously been suggested that dermal photoreceptor cells belong to a category of dispersed first order neurons, as found in classical retinal photoreceptors (Ramirez *et al.*, 2011). The only well-described phototransduction pathway for EOP in arthropods is in *Drosophila*, where in the larvae, predator avoidance is mediated by light detection in first order neurons which tile the body wall (Xiang *et al.*, 2010). The authors found that instead of using opsin molecules to sense light, a homologue of the *C. elegans* photopigment, lite1, known in *Drosophila* as Gr28b was required for light detection. Conversely, in *B. betularia* larvae, expression of classical visual genes such as opsin, arrestin, and RDB, suggests a similar visual cascade as in the arthropod eye, and that EOPs in *Drosophila* and *Biston* may have taken alternative evolutionary routes.

It was difficult to gain extracellular recordings from the ocelli, and even more so from dermal tissue in larvae, and this resulted in dermal recordings for only one individual. Although this does look like a real response to the light pulse, a larger sample size would be required, testing a larger range of colours. The difficulty in recording from dermal tissues was possibly due to the set-up, which was designed for eye recordings. Another problem was that we do not know precisely where in the dermis the putative photoreceptors are located. Molecular techniques such as Fluorescence in-situ hybridisation, as done in *Drosophila* larvae to target photoreceptor cells (Xiang *et al.*, 2010) could localise photoreceptors in the larval dermis.

It has been suggested that some visual genes have additional sensory functions other than phototransduction. For example, RDB in *Drosophila* is not only essential for normal phototransduction (Harris & Stark, 1977) but is also required for normal olfactory responses (Woodard *et al.*, 2007). One of the seven characterised opsins in *Drosophila*, Rh1, was found to be required for behavioural temperature discrimination, suggesting a thermosensory function (Shen *et al.*, 2011). Another study in a North American butterfly, *Limenitis arthemis*, has also suggested that opsins may play an alternative role to vision, perhaps in responses to temperature or photoperiod (Frentiu *et al.*, 2015). Opsins are very diverse, particularly in butterflies (Briscoe, 2008), and so it is not surprising that some gene variants may have alternative functions to phototransduction. Conversely, in moths, strong purifying selection was found in blue and LW opsins, suggesting that these genes have a conserved function of vision (Xu *et al.*, 2013). Although some of the known visual genes have been linked to other sensory pathways, it is highly unlikely that the expression of coding genes, such as opsins and RDB in the dermis of *B. betularia* larvae would not be involved in visual pathways that form part of the colour EOP mechanism.

## CONCLUSIONS

Expression of visual genes in dermal tissue in *B. betularia*, coupled with behavioural evidence, suggests that the colour change in larvae is aided by colour-sensitive EOPs located in the dermal tissue. It is possible that light detection in the skin may have originated from a protective function, whereby light stimulates melanin production, as found in many other species, including humans. Some of the gene variants expressed may still function in light avoidance/UV protection. Further investigation into these putative EOPs, for example, gene knockouts, successful dermal recordings using electrophysiology, and more behavioural data, would provide further evidence into the function of these genes. It would also be interesting to identify opsin homologues in other moth species and check for dermal transcripts to determine if this phenomenon is limited to *B. betularia*, or if it also occurs in other species of lepidopteran larvae.

# Chapter 6

## Extraocular opsin expression in Lepidoptera

**ABSTRACT**

Vision plays a universally essential role in nearly every species of animal, but the structure and sensitivity of visual systems is highly diverse, reflecting differences in selective forces and evolutionary constraints. In many animals, non-image-forming photoreception associated with circadian rhythm and UV protection occurs outside of the retina, in tissues such as the pineal organ and the brain. Dermal photoreception has been reported in a small number of taxonomically widespread species, and in *B. betularia* larvae, is associated with plastic colour change. This study collected opsin gene expression data for four opsin genes (UV, blue, LW1, LW2) in dermal tissue of adult and larval stages across 23 species of Lepidoptera and looked for associations with species phylogeny and a set of life history traits. Significant phylogenetic signals were detected for LW1 expression in larvae, and LW2 expression in adults. Larval colouration strategy and sex also affected opsin expression. Although the study requires data from more species, the results allude to possible non-retinal opsin functions in larvae and adults, and provide a baseline for further behavioural and genetic studies to untangle the complicated relationships between visual gene expression and function across the Lepidoptera. Understanding the phylogenetic relationships of opsin gene expression may help discover the evolutionary origin of dermal photoreception.

**INTRODUCTION**

The differences in visual systems between organisms have been shaped by the wide range of photic environments in which they live. Colour sensitivity, determined by specific amino acid residues of the opsin protein within the photopigment, is under selection from the ambient-light environment in which an organism lives (Yokoyama *et al.*, 1999). For example, fish inhabit some of the most diverse and challenging optical environments, resulting in high opsin diversity and expression patterns across fish species (Cummings, 2004; Dalton *et al.*, 2015). Visual signalling between conspecifics or predator-prey interactions may also drive colour vision (Lind *et al.*, 2017). Therefore, studying vision across species provides insights not only into the evolution of vision, but also the origins of the associated traits and behaviours.

The evolutionary relationship between colour vision and its associated traits has been frequently studied in birds and butterflies, due to a wealth of existing genetic and physiological data on vision, and because of the striking colours observed in these taxonomic groups (Hart *et al.*, 2000; Arikawa, 2003; Briscoe, 2008; Bloch, 2015).

The photoreceptors in papilionid and lycaenid butterflies have undergone several opsin duplication and diversification events, allowing for sensitivity to multiple wavelengths (Briscoe, 2008; Sison-Mangus *et al.*, 2008). In contrast, many moth species have retained the ancestral arrangement of three photoreceptors consisting of three opsin classes, sensitive to UV, blue and green (Lind *et al.*, 2017). However, duplication has also occurred in several opsin genes of the peppered moth, *B. betularia*, which are expressed in dermal tissue, as well as the eye (chapter 5). This result, coupled with behavioural data (chapters 3 and 4) suggests the presence of dermal photoreceptors, providing the larvae with additional information on the visual background to aid colour change.

Extraocular photoreception has been suggested as a way of mediating visual cues for colour change in lepidopteran pupae (Poulton, 1892; Angersbach, 1975), but other than our observations in *B. betularia*, there are no reports of extraocular photoreceptors (EOPs) in lepidopteran larvae. The evolutionary origins of EOPs are relatively unknown, as studies on this phenomenon have been limited to a few species across diverse taxonomic groups, with a range of different functions (Millot, 1968). Given the restricted research effort to date, it seems likely that the prevalence of EOPs is much higher than currently appreciated.

Much of the research on colouration in Lepidoptera is focused on the adult stage (Mallet & Joron, 1999; Siddique *et al.*, 2016; van't Hof *et al.*, 2016), but the larvae of this taxonomic group also display remarkable levels of colour variation, with functions ranging from mimicry and masquerade, to striking aposematism (Porter, 1997). The evolution of some of these larval colour patterns has been explained by life history traits, such as the association of masquerade with polyphagy (Higginson *et al.*, 2012). However, plastic colour change, as exhibited by *B. betularia* larvae is underreported, with only a few studies on other species (Grayson & Edmunds, 1989; Yamasaki *et al.*, 2009). General insights into the evolution of colour vision and potential for EOP in lepidopteran larvae may lead to descriptions of colour change and its association with EOP in other species.

When exploring colour vision, it makes sense to begin with opsins, as colour vision in Lepidoptera is primarily mediated by opsin-based visual pigments (Briscoe, 2000; Briscoe & Chittka, 2001). Differences in opsin expression could either reflect differences in abundance of photoreceptor type or the abundance of visual pigments

in each photoreceptor type; this in turn may affect colour discrimination thresholds, colour sensitivity, and speed of response (Calvert *et al.*, 2001; Vorobyev *et al.*, 2001). Opsin gene expression has been found to show greater evolutionary flexibility than opsin coding sequence (Bloch, 2015), and dermal EOP is regularly characterised by opsin expression (Okano *et al.*, 2000; Chen *et al.*, 2013; Ramirez & Oakley, 2015), making this a logical trait to explore the evolutionary patterns of non-retinal vision in Lepidoptera.

The aim of this study was to measure opsin expression in dermal tissue across both lepidopteran larvae and adults, to determine the potential for EOP in other lepidopteran species and provide insight into the evolutionary origin and routes of dermal opsins in *B. betularia*. Possible associations of opsin presence/expression with life history traits such as colour plasticity/crypsis *vs.* warning colouration may provide more complete understanding of the function of opsins.

## METHODS

### Sample collection

The species to be included in the dataset were chosen based on ease of collection; if they could be reared or wild-caught in the UK, whilst also ensuring a broad taxonomic range and including species with varied forms of larval colouration. Imagines were collected using a mercury vapour UV light trap, from various locations around Shropshire and Cheshire. Larvae were collected from hedgerows and trees in the same region. Some species, such as the cacao moth (*E. elutella*), Indian meal moth (*P. interpunctella*), and cotton bollworm (*H. armigera*) were obtained from lab-reared stocks. *B. mori* were purchased as eggs from Worldwide Butterflies and also lab reared. After identification, individuals were euthanised at -80$^{o}$C and stored until required. Larvae were euthanised in the same way as imagines, once they had reached final instar. This required any individuals that were not at final instar when caught to be lab reared until final instar.

As some opsin genes are involved in circadian rhythm, all tissue was frozen in daylight hours, around 2pm. Larvae and imagines were dissected into four parts: head, thorax, and abdomen, plus claspers in larvae and genitalia in imagines, as described previously (chapter 5, Fig. 5.1). As much internal tissue as possible, including guts, were removed and discarded, leaving only dermal tissue remaining.

**Gene expression**

Presence or absence of expression of each opsin gene (UV, blue, LW1, LW2) was determined using end-point RT-PCR for larval and adult head and dermal tissue (Fig. S6.1). RNA extraction and cDNA synthesis was also performed as described in chapter 5 (methods). For end-point PCR primer design (Table S6.1), opsin DNA sequences (UV, blue, LW1, LW2) for lepidopteran species were retrieved using the BLAST tool on NCBI (Altschul *et al.*, 1990), using *B. betularia* opsin DNA sequences as the search query. For species where opsin sequences were not available, tBLASTn searches were performed on WGS, using translated protein sequences of known opsin sequences in Geneious v7.1.7 (Kearse *et al.*, 2012). Where neither sequence, nor WGS information was available, degenerate primers were designed using alignments of highly conserved sequences (Table S6.1). End-point PCR was conducted as described in chapter 5, but with primer concentration increased to 1μM from 0.4μM per primer, to maximise annealing efficiency for degenerate primers.

**Life-history data**

For each species and individual, life history information, such as polyphagy status, larval colouration type, and sex were recorded. Polyphagy and colouration type were assigned to each species using information from Porter (1997) and Higginson *et al.* (2012). Colouration was split into 5 categories: none/white (N), aposematic (A), fixed cryptic (FC), polymorphic cryptic (MC), and plastic cryptic (PC). Cryptic colouration was defined as brown/green. Twenty-three species in total from 7 families of Lepidoptera were included in the dataset (Table 6.1).

**Table 6.1.** List of species included in lepidopteran opsin expression study

| Latin name | Common name | Family | Superfamily |
|---|---|---|---|
| *Laothoe populi* | Poplar hawk | Sphingidae | Bombycoidea |
| *Smerinthus ocellata* | Eyed hawk | | |
| *Bombyx mori* | Silkworm | Bombycidae | |
| *Agrotis exclamationis* | Heart and dart | Noctuidae | Noctuoidea |
| *Diarsia mendica* | Ingrailed clay | | |
| *Ochropleura plecta* | Flame shoulder | | |
| *Euplexia lucipara* | Small angle shades | | |
| *Phlogophora meticulosa* | Angle shades | | |
| *Helicoverpa armigera* | Cotton bollworm | | |
| *Acronicta psi* | Grey dagger | | |
| *Euproctis similis* | Yellowtail | Erebidae | |
| *Arctia caja* | Garden tiger | | |
| *Phigalia pilosaria* | Pale brindled beauty | Geometridae | Geometroidea |
| *Biston betularia* | Peppered moth | | |
| *Odontopera bidentata* | Scalloped hazel | | |
| *Selenia dentaria* | Early thorn | | |
| *Abraxas grossulariata* | The magpie | | |
| *Ourapteryx sambucaria* | Swallowtailed | | |
| *Operophtera brumata* | Winter moth | | |
| *Plodia interpunctella* | Indian meal moth | Pyralidae | Pyraloidea |
| *Ephestia elutella* | Cacao moth | | |
| *Galleria mellonella* | Greater wax moth | | |
| *Bicyclus anynana* | Squinting bush brown | Nymphalidae | Papilionoidea |

**Statistical analysis**

To determine if opsin expression presence/absence is correlated with phylogeny among species, a phylogenetic tree of those species was first constructed using previously determined relationships (Sihvonen *et al.*, 2011; Regier *et al.*, 2012; Regier *et al.*, 2013). Presence/absence of each opsin gene transcript (UV, blue, LW1, LW2) was calculated across all tissues of larvae and adults separately; 0 if there was no expression across tissues, and 1 if any dermal tissue expressed opsin. The pattern of these binary traits was tested for phylogenetic signal by calculating the *D* statistic, using the 'phylo.d' function (Fritz & Purvis, 2010) in the R package Caper (Orme *et al.*, 2013). *D* values closer to 0 are highly conserved under a Brownian threshold model, and values closer to 1 show randomly distributed traits.

The proportion of opsins expressed out of the four tested was also calculated for larvae and adults of each species, to provide a semi-continuous value. Phylogenetic signal in proportion of opsins expressed was calculated using Blomberg's *K* statistic (Blomberg *et al.*, 2003), using the 'phylosig' function in the R package Phytools (Revell, 2012). Computed *K* values indicate whether the evolution of a trait either (a)

does not show a significant signal ($K=0$); (b) is more conserved than expected by chance ($K>0$); (c) is less conserved than expected under Brownian motion evolution (BM) ($0<K<1$); (d) is as conserved as expected under BM ($K=1$); or (e) is more conserved than expected under BM ($K>1$).

To test other predictors of presence/absence of each opsin, a generalised linear model (GLM) was performed with several physiological/ life history variables (Table S6.3) set as predictors, in R version 3.1.0 (R Core Team, 2014).

**RESULTS**

Dermal opsin expression is not universal across Lepidoptera, with no dermal opsin expression found in some species (Fig. 6.1). For example, across the sample of lepidopteran larvae in this study, *B. betularia* was the only species to express all four opsin classes (UV, blue, LW1, and LW2) in dermal tissue. The majority of species expressed only one opsin gene (Fig. 6.1), and three species did not show larval dermal expression of any of the four opsin genes (*A. exclamationis, E. ellutella, and P. interpunctella*).

Of the adults sampled, three species, including *B. betularia*, expressed all four opsin genes in dermal tissue (Fig. 6.1), and higher proportions of dermal opsins were found across adult Lepidoptera, compared to their larval counterparts. For example, the adults of many species expressed half or more of total opsins (Fig. 6.1). Phylogenetic signal was not found in the pattern of opsin proportion for larvae ($K$=0.18, $P$=0.18; Table S6.2) or adults ($K$=0.17, $P$=0.22; Table S6.2); the distribution of traits differed from random, but were still less conserved than expected under Brownian motion.

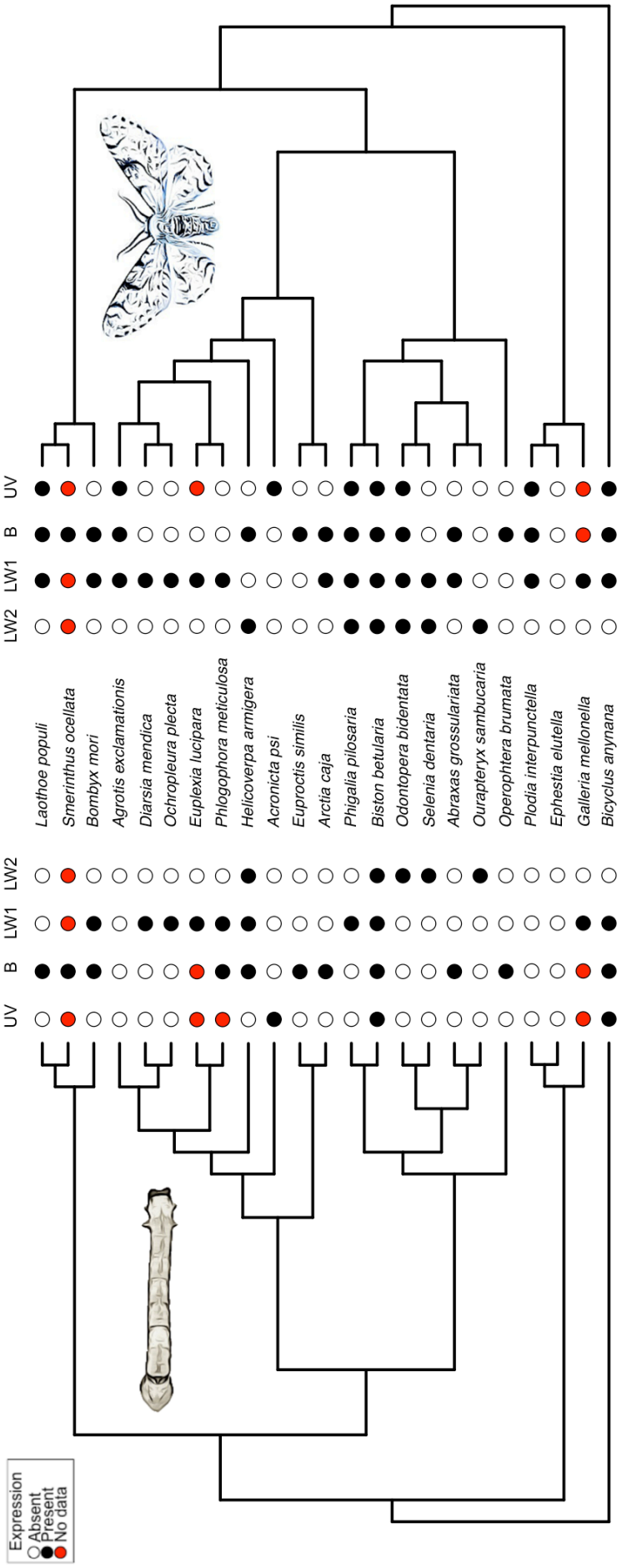


**Figure 6.1. Phylogenetic distribution of dermal opsin expression in the larval and adult stages of a diverse sample of Lepidoptera.** Presence/absence of dermal expression of opsin genes sensitive to ultraviolet (UV), blue (B), long wavelength copy 1 (LW1), and long wavelength copy 2 (LW2) mapped onto currently accepted phylogeny of Lepidoptera (Sihvonen *et al.*, 2011; Regier *et al.*, 2012; Regier *et al.*, 2013). Tree on left shows larvae, and adults are shown on the right, (presence = black, absence = white, and red = no data).

Within species, dermal expression differed between opsin classes in larvae and adults (Fig. 6.1). In larvae, UV opsin was expressed in three taxonomically diverse species across the lepidopteran phylogeny. Blue and LW1 expression was most prevalent across the sample taxa, with blue expression present in 11 of 23 species, and LW1 expressed in dermal tissue of 10 species (Fig. 6.1). LW2 opsin was expressed in dermal tissue of 5 species, all of these belonging to the family Geometridae, with the exception of one noctuid (*H. armigera*). LW1 opsin presence showed weak phylogenetic signal, as these traits differed significantly from a random distribution across the lepidopteran phylogeny ($P$=0.03; Table S6.2). UV, blue, and LW2 opsin distribution did not differ from random.

In adult dermal tissue, LW2 expression was least prevalent, found in only six species; five of these were from the family Geometridae, the remaining one from Noctuidae (Fig. 6.1). The pattern of LW2 expression was similar for adults as larvae, with the addition of *P. pilosaria* in adults, another geometrid species. In adults, blue and LW1 expression was most prevalent with 16 species expressing LW1 opsin, and 14 expressing blue opsin in dermal tissue (Fig. 6.1). The distribution of LW2 opsin in adults showed phylogenetic signal; it differed significantly from a random distribution across the lepidopteran phylogeny ($P$=0.01; Table S6.2). Distributions of dermal expression of UV, blue, and LW1 opsins across the lepidopteran phylogeny did not differ from random.

Dermal opsin expression was affected by a number of different life-history factors; for example, stage (larvae or adult), sex (male or female), and colouration type (aposematic, cryptic, or none) of the individual appeared to be predictors of dermal opsin expression (stage: $P$<0.0001, sex: $P$=0.01, colour: $P$<0.001; Table S6.3). Opsin gene class (UV, blue, LW1, or LW2) also affected presence or absence state ($P$=0.02; Table S6.3). Tissue type (thorax, abdomen, genitalia) did not significantly affect general opsin expression, nor did polyphagy status (tissue: $P$=0.50, polyphagy: $P$=0.45; Table S6.3).

**DISSCUSSION**

Dermal opsin expression is not restricted to the peppered moth, but is also not universal across Lepidoptera, where the phylogenetic pattern of expression is complicated, and appears to be affected by many different factors. In Lepidoptera, both the proportion of opsin genes expressed and presence/ absence of gene

expression showed weak to no phylogenetic signal for the majority of opsin genes (UV, blue, LW), with the exception of LW1 in larvae and LW2 in adults, where presence/ absence of gene expression deviates from random. The regulation of gene expression has been shown to be at the basis of adaptive phenotypic evolution in a wide range of taxa (Fay & Wittkopp, 2008), and may evolve through alterations in transcription factor deployment and/or cis-regulatory sequences (Carroll, 2005). Therefore, assuming that opsin transcript levels reflect protein abundance (Schwanhausser *et al.*, 2011), we also expected to see phylogenetic patterns in opsin gene expression among species of Lepidoptera.

The results we observed could be due to a number of different reasons; firstly, dermal expression in opsins LW1 and LW2 might show phylogenetic signal because they have a conserved function in dermal tissue, which selection is acting on. In contrast UV and blue may be under relaxed selection, as found in blue-sensitive opsins in fig pollinators (Wang *et al.*, 2013). Genes without a function should evolve neutrally, because without one, mutations cannot be deleterious or advantageous (Kalinka *et al.*, 2010). For example, in a multi-gene expression study in *Drosophila*, genes that conformed most to the expected pattern of divergence were genes involved in key developmental processes, whereby selection acts to conserve these patterns of gene expression (Kalinka *et al.*, 2010). This theory is corroborated by gene expression data on *B. betularia* (chapter 5), where, in larvae, dermal expression of LW1 opsin is upregulated to the level found in the eye, and the same was observed for LW2 opsin in adult *B. betularia* individuals.

The function of dermal photoreception in Lepidoptera is not yet fully understood, but in *B. betularia* larvae, it is thought to aid in colour change (chapter 3). Therefore, dermal photoreception may also function in colour change in other lepidopteran larvae. In support of this argument, larval colouration strategy was a predictor of dermal opsin expression, with cryptic species showing higher chance of dermal opsin expression, compared to those with aposematic or no colouration. This result may have been influenced by LW1 expression in larvae. Dermal LW1 expression was present in the larvae of *B. betularia* and its sister species, pale brindled beauty (*Phigalia pilosaria*), as well as a clade of noctuid species, including the cotton bollworm (*H. armigera*), ingrailed clay (*D. mendica*), angle shades (*P. meticulosa*), and flame shoulder (*O. plecta*). All of these species show some level of colour variation, suggesting that LW1 may have co-evolved with the capacity for colour

change in lepidopteran larvae. For example, *Helicoverpa armigera* larvae change colour, which is thought to aid crypsis on different parts of host plants (Yamasaki *et al.*, 2009), and although colouration has not been explicitly studied in *D. mendica*, *O. plecta*, or *P. meticulosa*, the larvae do appear to show colour variation (Porter, 1997). However, there is no conclusive evidence to determine whether the colour variation in the larvae of these species is plastic, or genetically determined and this information is unknown for the majority of lepidopteran larvae. After rearing these species for the purpose of this study, I have observed potential colour plasticity, but further exploration is required to understand whether visual cues induce colour change. This would enable clearer associations to be made between larval colour change and opsin expression across lepidopteran larvae.

Other predictors that affect dermal opsin expression may have confounded phylogenetic signal for blue and UV opsins; for example, in our study, sex and stage affected dermal opsin expression across species. It is not surprising that larvae and adults showed differential dermal opsin gene expression, as these two life stages are morphologically diverse and experience very different selective pressures. As a result of these differential selective forces, the larvae and adults of many holometabolous insect species show differences in eye morphology (simple vs. compound) and opsin gene expression (Pollock & Benzer, 1988; Henze *et al.*, 2012). In my study, life stage was controlled for, as phylogenetic analyses were conducted on larvae and adult data separately. Sex differences in opsin expression are not uncommon, particularly among colourful taxa, such as birds, fish, and butterflies, which are thought to be correlated with sexually dimorphic colouration, aiding mate recognition and choice (Sison-Mangus *et al.*, 2006; Laver & Taylor, 2011; Bloch, 2015). Adult swallowtail butterflies possess genital photoreceptors, proposed to aid in copulation and oviposition (Arikawa *et al.*, 1997). This highlights the diversity in function of EOPs between adult and larval stages of Lepidoptera, and may explain differential opsin expression found in the moth species of my study. Genital photoreceptors have not yet been identified in moths, but there is potential to investigate it, perhaps beginning with geometrid species that have been found to express dermal opsins.

It is possible that the sex differences in opsin expression may have skewed this dataset, as it is near impossible to catch females of some species of moth in the wild. Interestingly, New World warblers showed sexual dimorphism in UV and shortwave

(blue) opsin genes, but not LW (Bloch, 2015). In Lepidoptera it was blue and UV genes that showed reduced phylogenetic signal, which may potentially be caused by sex-differences found only in blue and UV opsins, but this suggestion requires further exploration with an increased sample of individuals of each sex. It has also been suggested that opsin gene expression in New World warblers is affected by habitat and light conditions (Bloch, 2015), which has also been observed in guppies (Sakai *et al.*, 2016), cichlids (Nandamuri *et al.*, 2017), and moths (Xu *et al.*, 2013).

The majority of the species in my study are nocturnal, or crepuscular, flying in dim light conditions, with many of the larvae also being active at night, making it difficult to categorise them by light environment (Waring & Townsend, 2009). However, I found that two of the three species of larvae that expressed no dermal opsins (*P. interpuntella* and *Ephestia elutella*) typically reside in dark habitats, effectively submerged in the food medium. Although it may be expected that habitat use will follow some phylogenetic pattern, many species experience rapidly-changing habitats which may lead to rapid fluctuation in opsin expression, sometimes in as little as three days (Nandamuri *et al.*, 2017). A comparative study of visual gene expression in day vs. night flying moth species may help to further understand responses to light environment in Lepidoptera. Diet and age have also been found to affect opsin expression in moths (Xu *et al.*, 2013), which may have affected the results of my study. In lab-reared samples, adults were euthanised at the same age, but it was not possible to standardise age for wild-caught samples. All larvae were euthanised at final instar, so this effect was controlled for.

In addition to differences in opsin gene regulation, spectral sensitivity within and between species may be altered by differences in amino acid sequence, gene duplication, or even gene loss. Opsins in insects, and butterflies in particular, have undergone a variety of gene duplication events (Sison-Mangus *et al.*, 2008; Feuda *et al.*, 2016). Six species in my sample possess a second copy of the long wavelength (LW) opsin gene in their genome. All six of these species showed dermal expression of that second copy (LW2), with the exception of *P. pilosaria* larvae. There may be gene duplication in the sample that has gone undetected, due to a lack of genetic resources for many species of Lepidoptera. For example, it is unlikely that *H. armigera* is the only noctuid species with two copies of LW gene, but genetic resources predominantly exist for pest species (Perera *et al.*, 2016; Gouin *et al.*, 2017), and are scarce for other noctuid species. A closely related family to Noctuidae

and part of the Noctuoidea superfamily, is Erebidae, represented by the garden tiger (*Arctia caja*) in my sample. The day-flying scarlet tiger moth (*Callimorpha dominula*) is a sister species to the nocturnal garden tiger, and has evolved four copies of LW gene (Feuda *et al.*, 2016). Based on a single band in the LW opsin PCR, it has been assumed that *Arctia caja* has only one copy of LW. However, inspection of the genome or transcriptome would be required to see if LW gene duplication has also occurred in this species, or if differences in their photic environments have caused alterations in the number of LW opsin copies in these closely related species.

Although we can see plausible patterns in opsin expression across my sample of Lepidoptera, we should interpret the results of this study with caution. For example, we cannot conclude from a dataset of only 23 species of Lepidoptera that there is no phylogenetic signal in blue and UV genes. There may be other evolutionary forces affecting the patterns in opsin expression observed across phylogeny, such as stabilising selection, which can erode phylogenetic signal (Bedford & Hartl, 2009), and explains the expression patterns of several genes in *Drosophila* (Kalinka *et al.*, 2010). In reality, gene expression is not a binary trait, and by characterising it as such (owing to practical constraints) we have lost variation and therefore information between species. To overcome this limitation, the degree of gene expression would need to be quantified. However, Lepidoptera are generally a challenging taxonomic group to perform large-scale comparative studies due to patchy genomic resources and life history information.

## CONCLUSIONS

This study aimed to explore extraocular photoreception, by detecting opsin gene expression across Lepidoptera; to determine if dermal opsin is restricted to *B. betularia*, and if not, how it has evolved, and what might be driving it. The results indicate that patterns of dermal opsin expression in Lepidoptera are complex, but were affected by larval colouration type, which could also be related to plastic colour change, which has not yet been formally identified in many species. The differences in phylogenetic signal observed in long wavelength copies one and two provide evidence for alternative functions in the dermis of larvae and adults. This data provides a basis for future work to further explore spectral sensitivity and colouration patterns in Lepidoptera, for which there are presently huge gaps in the knowledge.

# Chapter 7
## General discussion

### General aims and findings

The purpose of this thesis was to provide a better understanding of morphological colour change and visual perception in larvae of the peppered moth (*Biston betularia*), in the context of anti-predator camouflage, and to assess the capacity for extraocular photoreception in Lepidoptera more generally. To achieve this, I combined a series of behavioural and genetic experiments. First, I measured the visual response, including the cues that *B. betularia* larvae were able to respond to and the extent of their colour change. Second, I examined the possibility of extraocular vision by testing the ability of blindfolded larvae to change colour compared to non-blindfolded controls, and measured the expression of visual genes in dermal tissue.

Results from these experiments showed that larvae responded to both colour and luminance cues separately to produce a continuous range of phenotypes. When blindfolded, larvae were still able to respond to, and produce phenotypes to match combinations of colour and luminance cues, as effectively as non-blindfolded controls. The phenotypic response, which implies a sophisticated system of extraocular photoreception, was corroborated by high expression of visual genes in the larval dermis compared to the head. As well as changing colour to match variable visual backgrounds, *B. betularia* larvae chose to rest on backgrounds that better matched their own colour. The pattern of dermal opsin expression across Lepidoptera differed between opsin genes and developmental stage, and was affected by larval colouration type.

### Colour change in *Biston betularia* larvae

By manipulating colour and luminance only, my experiments confirm that the phenotypic response observed in *B. betularia* larvae was to visual cues, as suggested previously by Noor *et al.* (2008). However, the continuous range of colour phenotypes produced by larvae was not in accordance with Noor's inference that the response was a phenotypic switch between brown and green (Noor *et al.*, 2008). Dynamic colour change has been observed across a wide range of taxa, for a variety of purposes, including communication (Stuart-Fox & Moussalli, 2008; Stuart-Fox &

Moussalli, 2009), thermoregulation (Trullas *et al.*, 2007; Vroonen *et al.*, 2012), and camouflage (Clarke & Schluter, 2011; Stevens *et al.*, 2014a; Kang *et al.*, 2016; Eacock *et al.*, 2017). It has been assumed that colour change in *B. betularia* larvae has evolved to improve concealment from predators, but colour had only been measured from a human perspective (Noor *et al.*, 2008). I modelled colour using the avian visual system and found that by changing colour, larvae became less conspicuous to avian predators, providing critical support for the view that this is an adaptation to increase predator avoidance, and therefore survival.

In the last decade camouflage research has shown an increase in objective, quantitative testing, allowing phenotypic transitions to be assessed in an evolutionary context. For example, colour change in frogs, sand fleas, and shore crabs (Stevens *et al.*, 2014b; Stevens *et al.*, 2015; Kang *et al.*, 2016) has been modelled from the visual perspective of potential snake and bird predators. However, there are still many examples of studies where colour change has been subjectively measured, or objectively modelled, but analysed from human perspective (Grayson & Edmunds, 1989; Yamasaki *et al.*, 2009).

The varied repertoire of colour patterns exhibited by many fish and cephalopod species are proposed to differ based on courtship or camouflage purposes (Watson *et al.*, 2014; Allen *et al.*, 2015). Similarly, in reptiles, it is sometimes uncertain whether colour change is occurring for the purpose of thermoregulation or camouflage (Smith *et al.*, 2016). In such cases, visual modelling, or empirical predator-prey experiments (Merilaita *et al.*, 2001) would enable more solid conclusions to be drawn about the purpose and evolution of colour change. Recent advances in molecular techniques have enabled many studies to explore the genetic and hormonal basis of phenotype changes in insects such as colour-changing spiders and swallowtail butterfly larvae (Futahashi & Fujiwara, 2008b; Futahashi & Fujiwara, 2008a; Llandres *et al.*, 2013). These data, combined with behavioural experiments or visual modelling would provide more complete information on the proximate and ultimate causes of dynamic camouflage systems.

**Extraocular photoreception in *B. betularia***

The majority of reports on dynamic camouflage are in animals that change colour rapidly using chromatophores, such as cephalopods, amphibians, and fish (Mathger & Hanlon, 2007; Skold *et al.*, 2013; Watson *et al.*, 2014). Chromatophores have been

found to express visual genes such as opsins (Chen *et al.*, 2013; Fulgione *et al.*, 2014; Kingston *et al.*, 2015), and behavioural experiments have provided further evidence for dermal photoreception in some of these animals (Pankey *et al.*, 2010; Fulgione *et al.*, 2014). Therefore, it was somewhat, but not completely surprising to find evidence for dermal photoreception in *B. betularia*. Colour change in *B .betularia* larvae is not likely to be mediated by chromatophores, instead it appears to occur from movement of pigmentation granules, known as morphological colour change (Buckmann, 1977; Insausti & Casas, 2008). This is one of the first examples of morphological colour change facilitated by extraocular photoreceptors, with the exception of two historical examples in butterfly pupae 'silvering' and melanisation (Poulton, 1892; Angersbach, 1975). Measuring opsin expression in larvae and pupae of butterfly species that show light-induced pupal melanisation could provide insight as to the visual mechanisms behind these phenotypic changes.

In *B. betularia*, dermal expression of opsins varied depending on gene and differed between larvae and imagines. Retinal degeneration B (RDB), a gene essential for the maintenance of photoreceptors was upregulated in both larvae and imagines of *B. betularia*, suggesting that dermal photoreception occurs in both stages. However, there was a strong contrast in the dermal: head expression patterns of the two long wavelength gene copies between larvae and adults. In dermal tissue LW1 was upregulated in larvae and downregulated in adults, and the opposite was found for LW2, which was downregulated in larvae and upregulated in adults. These patterns could indicate functional differences of the two gene copies. Additionally, the molecular phylogeny of a small sample of opsins in Lepidoptera suggests that LW1 and LW2 evolved as independent gene copies in *B. betularia* and two closely related species: scalloped hazel (*O. bidentata*) and pale brindled beauty (*P. pilosaria*), strengthening the evidence for separate functions in LW1 and LW2.

Although the blindfolding experiments in larvae reveal a potential function in colour change for dermal photoreceptors, for the adult moths, which display fixed phenotypes (van't Hof *et al.*, 2016), the function is currently unknown. There are two plausible explanations for dermal photoreception in adult *B. betularia*: 1) behavioural background matching, and/or 2) genital photoreceptors for copulation and oviposition. Genital photoreceptors have been discovered in butterflies (Arikawa & Miyako-Shimazaki, 1996; Arikawa *et al.*, 1997), but this possibility has not yet been explored in moths. Evidence is limited, but adult peppered moths have been

reported to select backgrounds that better match their own colour (Grant & Howlett, 1988). It is therefore possible that dermal photoreceptors assist in background choice.

Microhabitat choice experiments revealed that *B. betularia* larvae rested on colours that would increase their crypticity through dermal photoreceptors, but only when the two options differed in luminance as well as colour. Although *B. betularia* larvae respond semi-independently to colour and luminance cues to change colour, microhabitat choice may require additional luminance cues. Many examples of colour change or habitat choice are in response to luminance only, or there is a stronger response to luminance cues (Stevens *et al.*, 2014b; Kang *et al.*, 2016; Polo-Cavia & Gomez-Mestre, 2017). It may seem unnecessary for a colour-changing animal to select backgrounds to increase crypsis, but this behaviour may have evolved in response to slow, imperfect colour change. In the wild, *B. betularia* larvae move between neighbouring twigs, which show high colour heterogeneity. Colour change may not occur quickly enough to keep up with this movement, leaving larvae vulnerable to predation. Relaxed selection on colour change may have occurred in response to multiple defences in *B. betularia* larvae. Masquerade provides additional protection from predation in peppered moth larvae (Skelhorn & Ruxton, 2010). There is evidence for chemical crypsis in a sister species, *Biston robustum*, whereby the larval stages mimic the chemical signatures of their food plant to avoid detection by predators through olfactory signals (Akino *et al.*, 2004). *B. betularia* may also use chemical crypsis as an additional defense mechanism.

Due to the position of larval ocelli in relation to the rest of the body and the daytime resting position of *B. betularia* larvae, where their ocelli are somewhat distant from the twig, dermal photoreceptors may be required in *B. betularia* larvae to provide more complete information on twig colour and pattern than can be achieved with the ocelli alone. This information could then potentially be used to increase crypsis, either physiologically through colour change, or behaviourally through behavioural background matching. If larvae are processing visual signals from photoreceptors in both the dermis and ocelli, this signal would need to be integrated to produce the target colour in the dermis and movement towards more similarly-coloured twigs. For colour change, there is likely some kind of neural feedback loop between colour perception and pigment movement for colour change. This could be localised to dermal tissue, as it is in chromatophores, which can change colour in response to

light when isolated from the rest of the animal (Daniolos *et al.*, 1990). This may allow different segments of the larval dermis to change colour independently to one another, providing an explanation for the observation of stripes when larvae were placed on striped dowels (Fig. 7.1). However, for visual information to elicit changes in behaviour, this information is usually processed in the brain. The fact that blindfolded larvae were still able to select resting substrates more closely matched in colour suggests that the signal from dermal photoreceptors is integrated in the CNS.

It still remains elusive as to whether colour change in *B.betularia* larvae, and perhaps other species drove the evolution of dermal photoreception, or whether it was originally selected for in the adult moths. Further molecular analysis into LW gene duplication in a larger sample of species is required to explore this. The species-wide opsin phylogeny (chapter 6) suggests that dermal opsin expression may not have existed as an ancestral state for the majority of opsin genes. However, this cannot be confirmed without the addition of more primitive moth species.

**Dermal opsin expression across Lepidoptera**

To determine if extraocular photoreception was restricted to *B. betularia*, I measured dermal opsin expression in a subset of species across Lepidoptera and explored any potential phylogenetic signal of opsin expression. The highly variable pattern of dermal opsin expression, largely lacking phylogenetic signal, was unexpected and contrasts with ocular opsin expression in birds and cichlids, which is highly conserved and shows strong phylogenetic pattern (O'Quin *et al.*, 2010; Bloch, 2015). However, as the condition of my multi-species sample could not be strictly controlled, the phylogenetic signal may have been partially masked by environmental effects, which have been known to affect visual gene expression (Yan *et al.*, 2014; Dalton *et al.*, 2015; Nandamuri *et al.*, 2017). It was interesting that the phylogenetic signal observed correlated with high dermal expression (LW1 in larvae and LW2 in adults). Not all lepidopteran genomes contain a second copy of long wavelength opsin, and the second copy may have evolved in certain species for dermal functions, but this requires further exploration. The differential pattern of expression and phylogenetic signal between larvae and adults could suggest alternative dermal functions for LW1 and LW2; colour change in larvae and perhaps genital photoreception or microhabitat choice in adults. LW genes over blue or UV would be expected in such functions, as they require sensitivity to colours in the long

range of the visual spectrum, such as green and brown. These colours are more commonly found in habitats of *B. betularia* and other Lepidoptera (Porter, 1997).

The association between larval colour pattern and dermal expression across opsins is also interesting, as the possible coevolution of colour vision and colouration in animals has been a topic of extensive scientific discussion (Lind *et al.*, 2017). There is some evidence for coevolution of colour signals and colour vision within communication systems (Ryan & Cummings, 2013; Bloch, 2015). However, lepidopteran larvae are not reproductive and do not signal to conspecifics, and so their colouration is more likely to coevolve with the colour vision of their predators (Geisler & Diehl, 2003; Blount *et al.*, 2009). Colouration patterns in larvae may be associated with habitat type and behaviour; for example, larvae with no colouration, such as many species of Pyralidae, live in dark habitats such as grain stores or beehives (Mohandass *et al.*, 2007; Kwadha *et al.*, 2017). Aposematic animals behave differently to those with cryptic colouration (Despland & Simpson, 2005; Ioannou & Krause, 2009) and aposematic larvae may rest in more exposed areas, whereas cryptic animals may hide in shaded areas. Environmental light intensity is known to affect opsin expression (Dalton *et al.*, 2015; Sakai *et al.*, 2016; Nandamuri *et al.*, 2017), and so the effect of colouration type on dermal opsin expression in Lepidopteran may actually be caused by habitat type; for example, if *P. interpunctella* and *E. elutella* were reared in more transparent medium and exposed to light, upregulation of dermal opsin expression may have been observed. Further investigation of life history factors, such as habitat and how these factors interact in Lepidoptera is required to understand the evolution of dermal photoreception, as well as extending this study with more representative species from each taxonomic group

**Implications and future direction**

Finding evidence for dermal photoreception in the larvae of the peppered moth has important implications for camouflage and vision, both of which are fundamental for animal survival and have shaped evolution in many species. This is the first example of colour change mediated by dermal opsin expression in insects, and therefore opens the possibility to investigate colour change and potential extraocular photoreception in other species of Lepidoptera. This may provide insight into the evolution of camouflage and visual systems more generally in the animal kingdom. As well as being an exciting branch of discovery science, colour change research has potential applications for pest control. As discussed previously, colour change and

dermal photoreception may have also evolved in the Noctuidae. Many noctuid species are major crop pests and if more is known about their camouflage, this may help in controlling these species.

Although this research has answered many questions regarding colour change and photoreception in peppered moth larvae and other species of Lepidoptera, there are still many questions left unanswered. Future work could explore the molecular mechanism of colour change in *B. betularia* larvae through transcriptomics, metabolomics, or hormone analysis. These techniques have been applied to other species of lepidopteran larvae, where juvenile hormone and several genes have been identified in colour change (Hori & Riddiford, 1982; Futahashi & Fujiwara, 2008b; Futahashi & Fujiwara, 2008a). Molecular techniques such as *in situ* hybridisation (ISH) may also be useful to determine the exact location of opsin expression in larval dermal tissue, as demonstrated in a study on *Drosophila* larvae (Xiang *et al.*, 2010). I attempted ISH experiments with RNA probes, but fluorescent markers were required for visualisation in melanised dermal tissue.

Further exploration of the visual system in larvae may increase our understanding of colour change in Lepidoptera. This could include measuring the expression of additional visual genes, or modelling of colour from the visual perception of larvae. Spectral sensitivities for visual modelling in larvae could be gained by intracellular recordings of the ocelli in response to different light wavelengths and intensities. Although limited, the extracellular dermal recordings showed possible evidence for a response to light and so further electrophysiological experiments, with a more specialised set-up may provide more conclusive evidence for neural responses to light, in the dermal tissue of *B. betularia* and perhaps other Lepidoptera. Further behavioural experiments on larval vision could include decreasing light intensity to find how this affects the ability to perceive colour; it would be interesting to test the ability for colour vision in *B. betularia* larvae at low light levels. Generally, much more information on the life-history of Lepidoptera and an increase in genomic resources is required to continue to investigate visual perception and colour change in lepidopteran larvae, and the functional value of dermal photoreceptors in adult moths and butterflies. Measuring dermal opsin expression in more butterfly species may prove interesting, as there is behavioural evidence for dermal photoreception in these taxa (Poulton, 1892; Angersbach, 1975; Arikawa *et al.*, 1997).

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# Supplementary information

**Table S2.1.** Experimental design summary for dowel experiments conducted in chapter 2

| Treatment name and label | Treatment | Dowel paint (Dulux) | Number replicate boxes | Adult melanic morph genotypes* | Sample size |
|---|---|---|---|---|---|
| **Isoluminant dowels** | | | | | |
| IB | Isoluminant brown | Wild mushroom 1 | 5 | One family: *t/t*, *i/t* | 61 |
| IG | Isoluminant green | Indian ivy 2 | 5 | | 66 |
| **Luminance gradient** | | | | | |
| Bl | Black | Night jewels 1 | 3 | Two families: *t/t* | 20 |
| BW1 | Dark grey | Night jewels 2 | 3 | | 11 |
| BW2 | Mid grey | Grey steel 1 | 3 | | 17 |
| BW3 | Light grey | Grey steel 2 | 3 | | 9 |
| Wh | White | Chiffon white 4 | 3 | | 20 |
| **Chroma and luminance gradient** | | | | | |
| Br | Brown | Espresso shot | 3 | Four families: *t/t*<br>One family: *t/t*, *c/t*, *c/c* | 42 |
| BG1 | Brown-green 1 ‘more brown’ | 25:75 Indian ivy 3: Espresso shot | 3 | | 67 |
| BG2 | Brown-green 2 ‘50:50 brown green’ | 50:50 Indian ivy 3: Espresso shot | 3 | | 67 |
| BG3 | Brown-green 3 ‘More green’ | 75:25 Indian ivy 3: Espresso shot | 3 | | 70 |
| Gr | Green | Indian ivy 3 | 3 | | 34 |
| **Heterogeneous dowels** | | | | | |
| 0G | Heterogeneous dowels – 100% brown | Espresso shot | 1 | Two families: *t/t* | 19 |
| 30G | Heterogeneous dowels- 30% green, 70% brown | Indian ivy 3 Espresso shot | 1 | | 23 |
| 50G | Heterogeneous dowels- 50% green, 50% brown | Indian ivy 3 Espresso shot | 1 | | 19 |
| 70G | Heterogeneous dowels- 70% green, 30% brown | Indian ivy 3 Espresso shot | 1 | | 21 |
| 100G | Heterogeneous dowels – 100% green | Indian ivy 3 | 1 | | 22 |

***** Expected genotypes of $F_1$ larvae at the locus that determines the adult morph, based on known genotypes of the parents (alleles: *t* = *typica*; *i* = *insularia*; *c* = *carbonaria*).

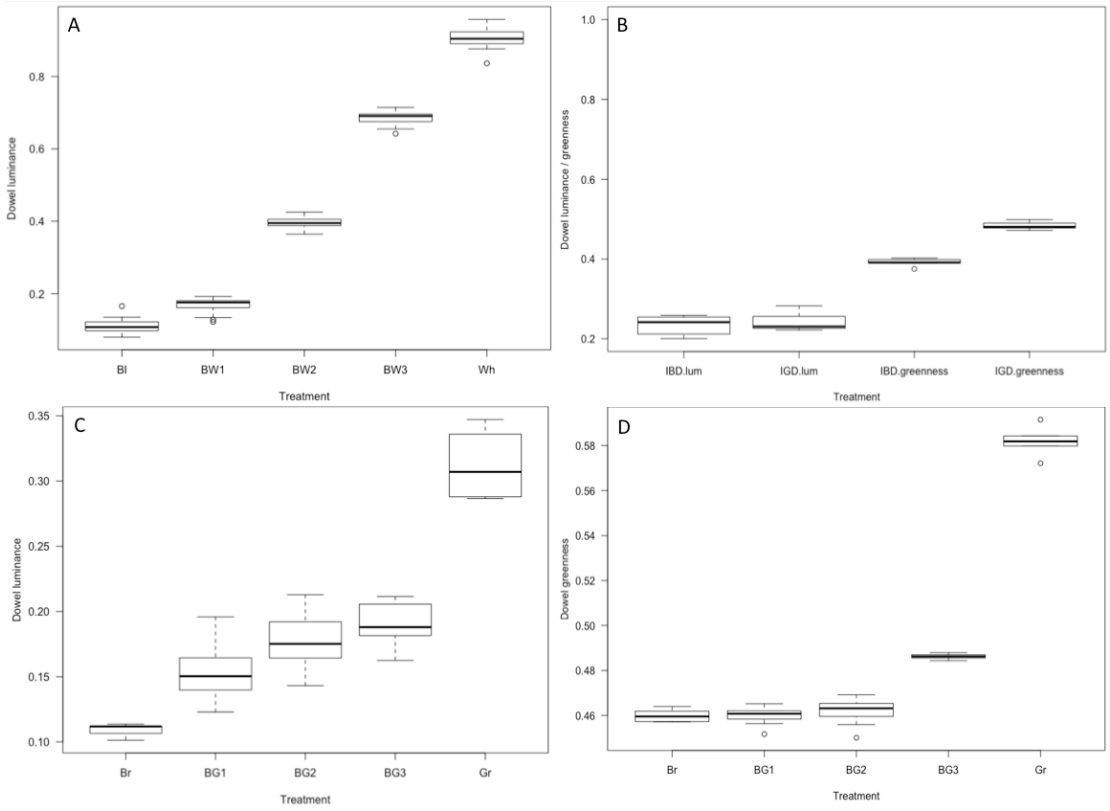


**Figure S2.1. Distribution of dowel luminance and greenness. (**A) Luminance of dowels used in luminance gradient experiment; (B) Luminance and greenness of isoluminant brown (IBD) and isoluminant green (IGD) dowels; (C) Luminance of dowels used in colour and luminance gradient experiment; (D) Greenness of dowels used in colour and luminance gradient experiment. For explanation of treatment codes see Table S2.1.

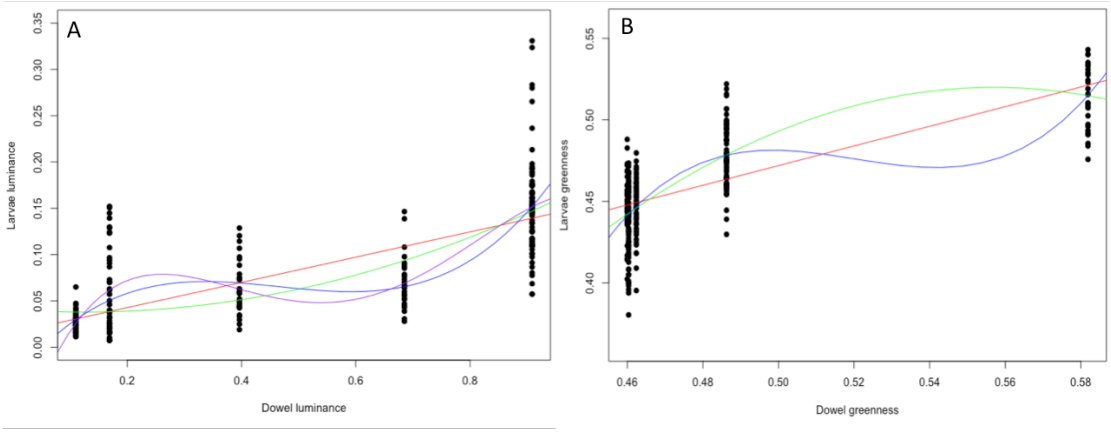


**Figure S2.2. Polynomial model fitting of larvae luminance and greenness in response to dowel gradient treatments.** (A) Scatterplot of dowel and larvae luminance from luminance experiment. (B) Scatterplot of dowel and larvae greenness from colour experiment. Polynomial models represented in both panels (A) and (B) by colours: 1$^{st}$ order (red), 2$^{nd}$ order (green), 3$^{rd}$ order (blue), 4$^{th}$ order (purple).

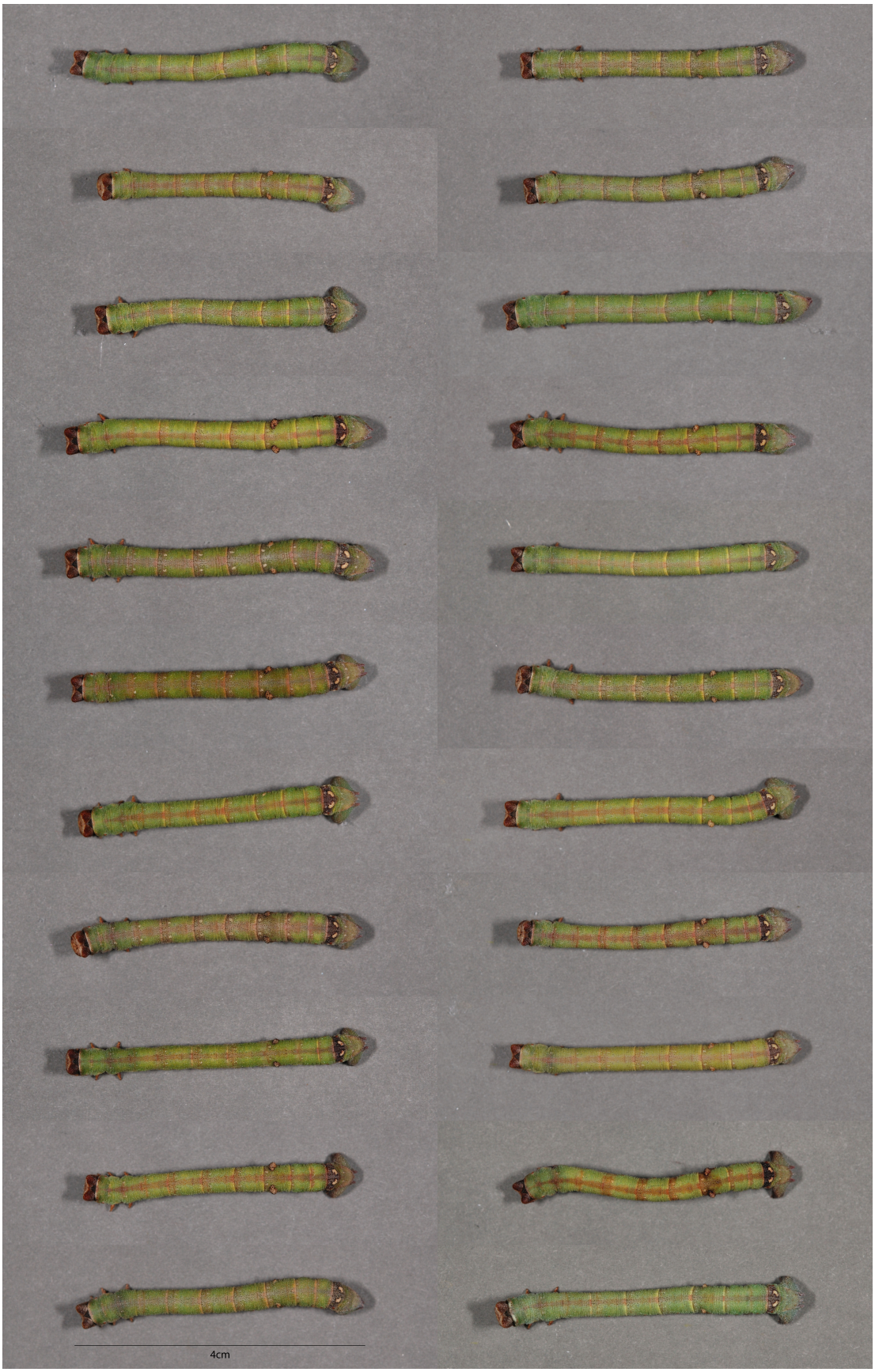


**Figure S2.3A. Heterogeneous environment treatment**. Photographs of the final instar *B. betularia* larvae under treatment 100G (100% Green, 0% brown dowel proportions).

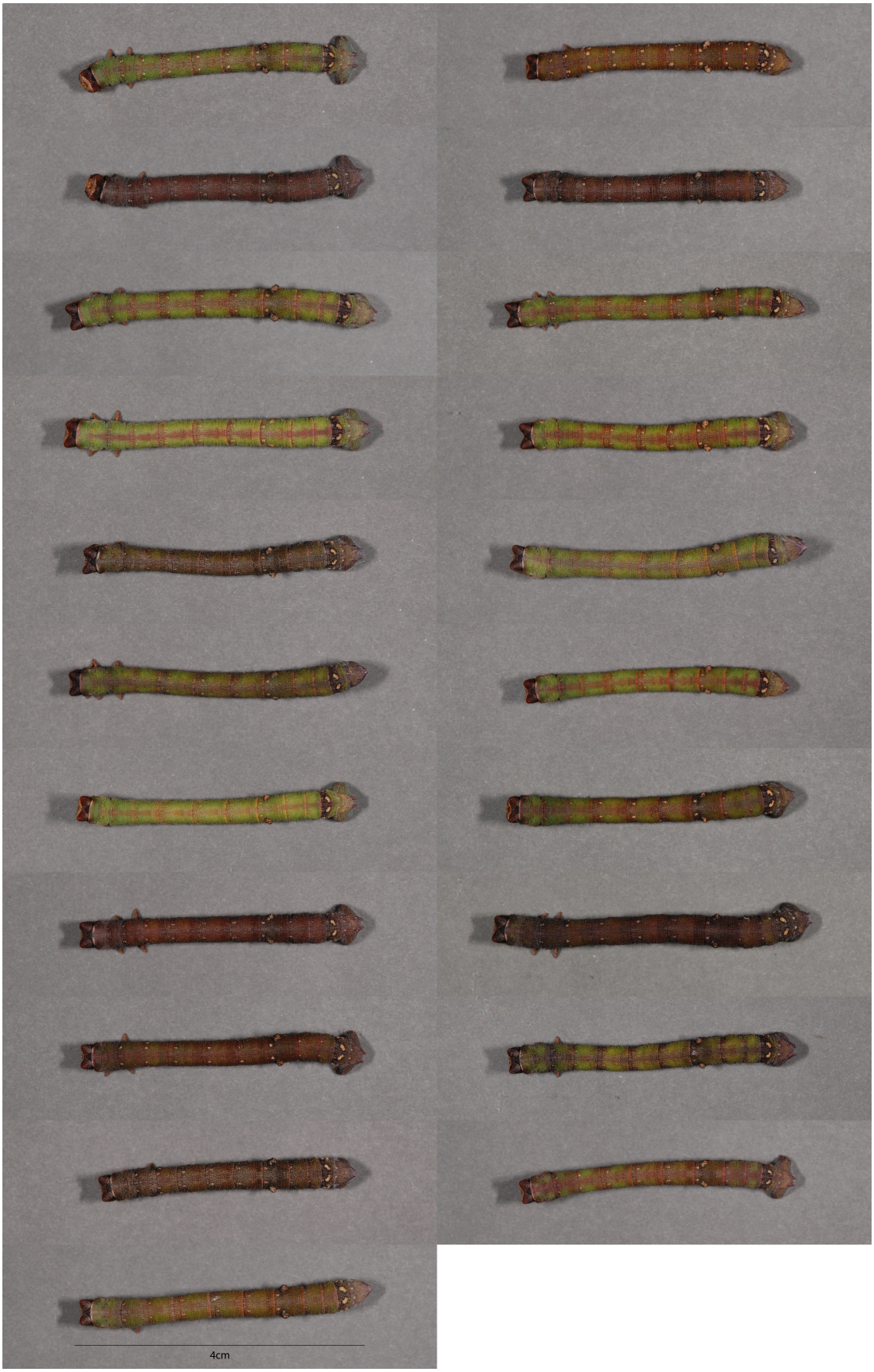


**Figure S2.3B. Heterogeneous environment treatment**. Photographs of the final instar *B. betularia* larvae under treatment 70G (70% Green, 30% brown dowel proportions).


**Figure S2.3C. Heterogeneous environment treatment**. Photographs of the final instar *B. betularia* larvae under treatment 50G (50% Green, 50% brown dowel proportions).


**Figure S2.3D. Heterogeneous environment treatment**. Photographs of the final instar *B. betularia* larvae under treatment 30G (30% Green, 70% brown dowel proportions).


**Figure S2.3E. Heterogeneous environment treatment**. Photographs of the final instar larvae under treatment 0G (0% Green, 100% brown dowel proportions).

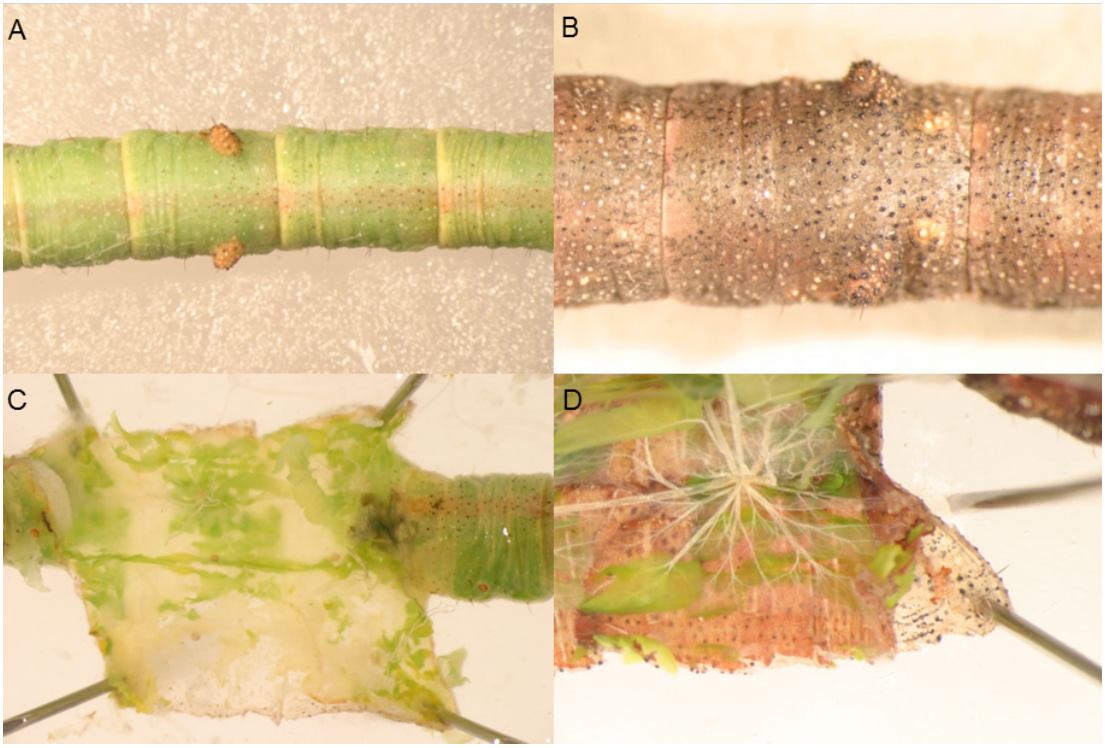


**Figure S2.4. The external colour of *B. betularia* larvae is achieved by a three-layer palette.** External dorsal surface of green (A) and brown (B) larvae. Dissection of the same larvae reveals that the primary colour in green phenotypes comes from underlying fatty tissue visible through translucent cuticular and epidermal layers (C). In brown phenotypes, there is less green tissue, the epidermis is reddish brown, and the cuticle has pronounced black spots (D).

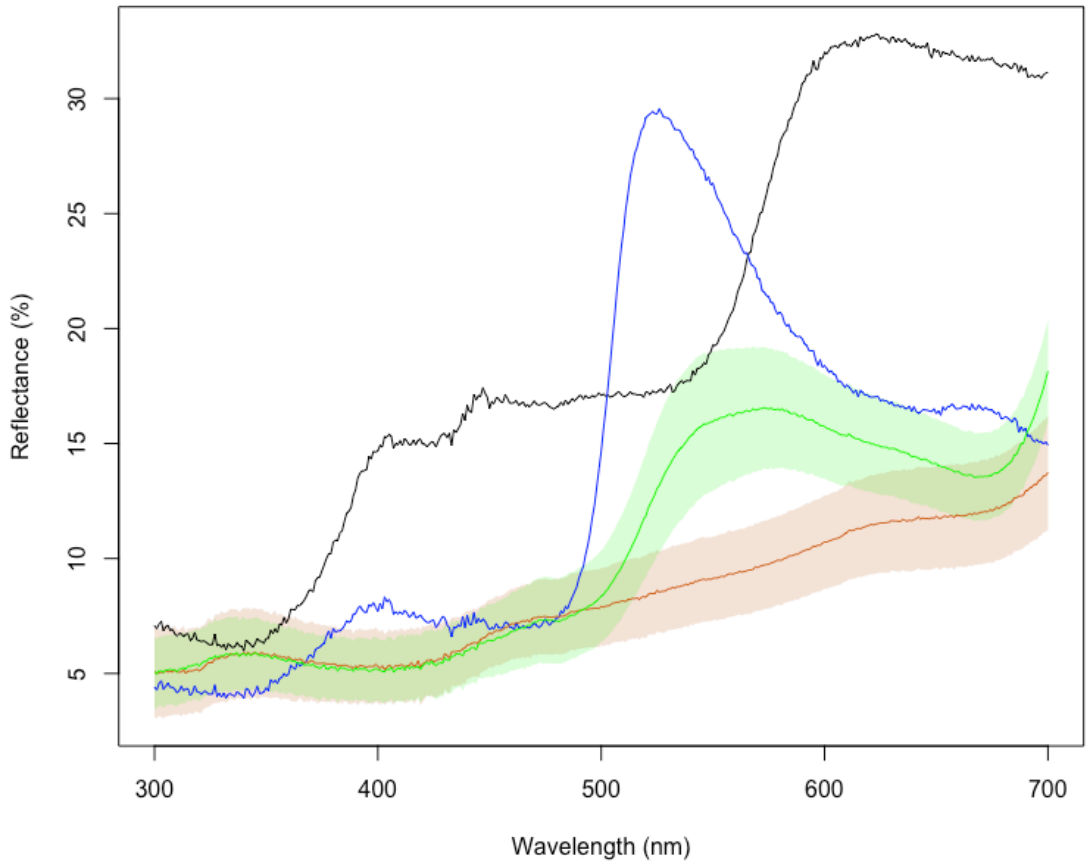


**Figure S2.5. Raw reflectance spectra of isoluminant *B. betularia* larvae and dowels.** Spectra shown in visible wavelength range (300-700 nm), where brown = brown larvae (*n*= 61), green= green larvae (*n*= 66), black= brown dowel, blue= green dowel. Shading around larvae represents standard errors between individuals.

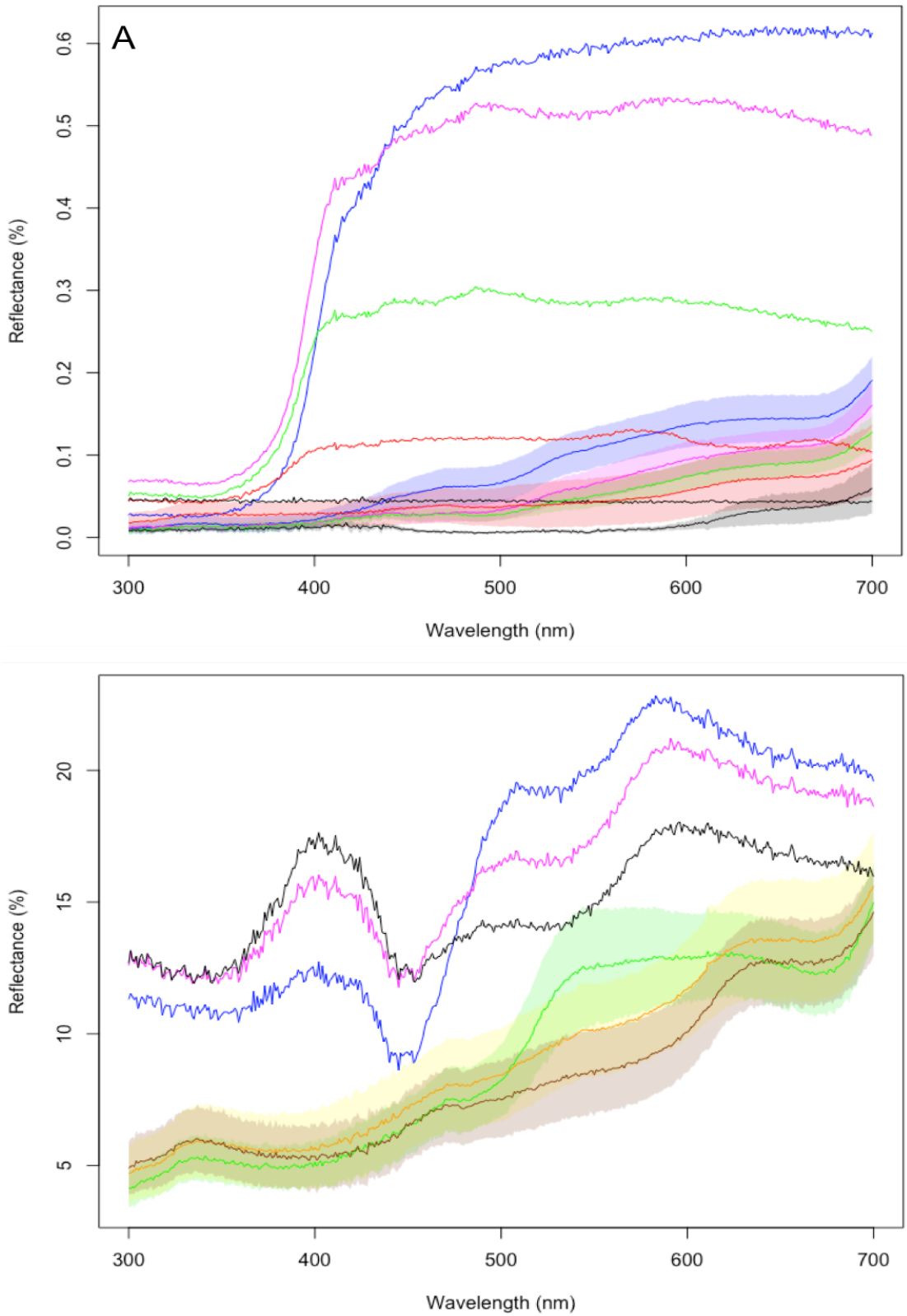


**Figure S2.6. Raw reflectance spectra of reaction norm *B. betularia* larvae and dowels.** Spectra shown in visible wavelength range (300-700 nm), (A) luminance gradient, where black = black (Bl: *n*= 20), red= dark grey (BW1: *n*= 11), green= mid grey (BW2: *n*= 17), magenta= light grey (BW3: *n*= 9), and blue= white (Wh: *n*= 20) for both larvae (with shading) and corresponding dowels. (B) Colour reaction norm where black= 'more brown' (BG1) dowel, magenta= '50:50 brown-green' (BG2) dowel, blue= 'more green' (BG3) dowel, brown= BG1 larvae, orange= BG2 larvae, green= BG3 larvae. Shading around larvae represents standard errors between individuals.


**Figure S3.1. Design of treatment arenas.** (A) Brown dowel arena with final instar larvae; (B) green dowel arena with final instar larvae.

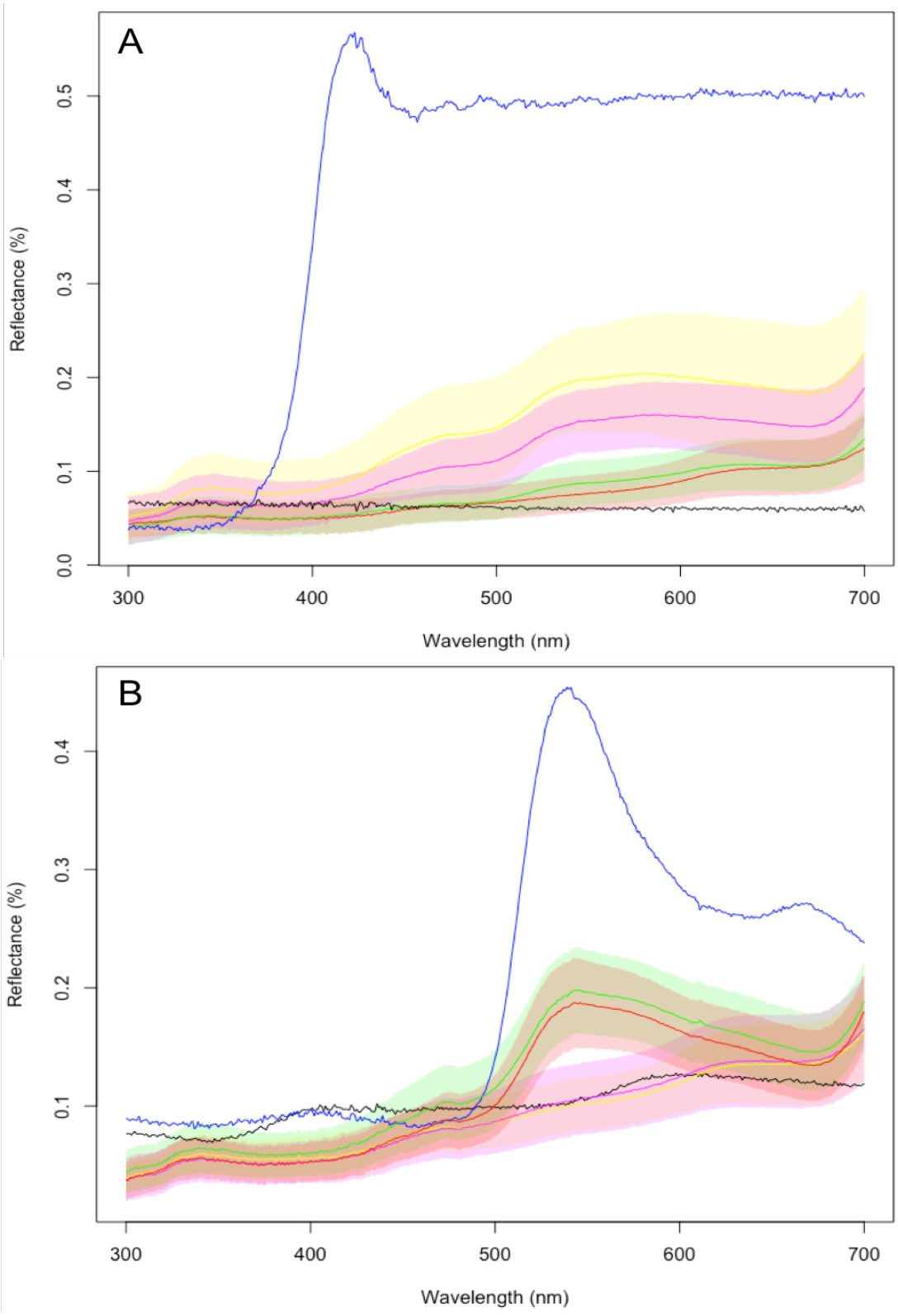


**Figure S3.2. Raw reflectance spectra of *B. betularia* larvae and dowels from blindfolding experiments.** Spectra shown in visible wavelength range (300-700 nm). (A) Black and white blindfolding experiment, where black = black dowel (BLD), blue= white dowel (WD), red= black control larvae (BLC: *n*= 29), green= black blindfolded larvae (BLP: *n*= 45), yellow= white control larvae (WC: *n*= 26), magenta= white blindfolded larvae (WP: *n*= 49). (B) Brown and green blindfolding experiment, where black= brown dowel (BD), blue= green dowel (GD), yellow= brown control larvae (BC: *n*= 44), magenta= brown blindfolded larvae (BP: *n*= 50), green= green control larvae (GC: *n*= 36), and red= green blindfolded larvae (GP: *n*= 31). Shading around larvae represents standard errors between individuals.

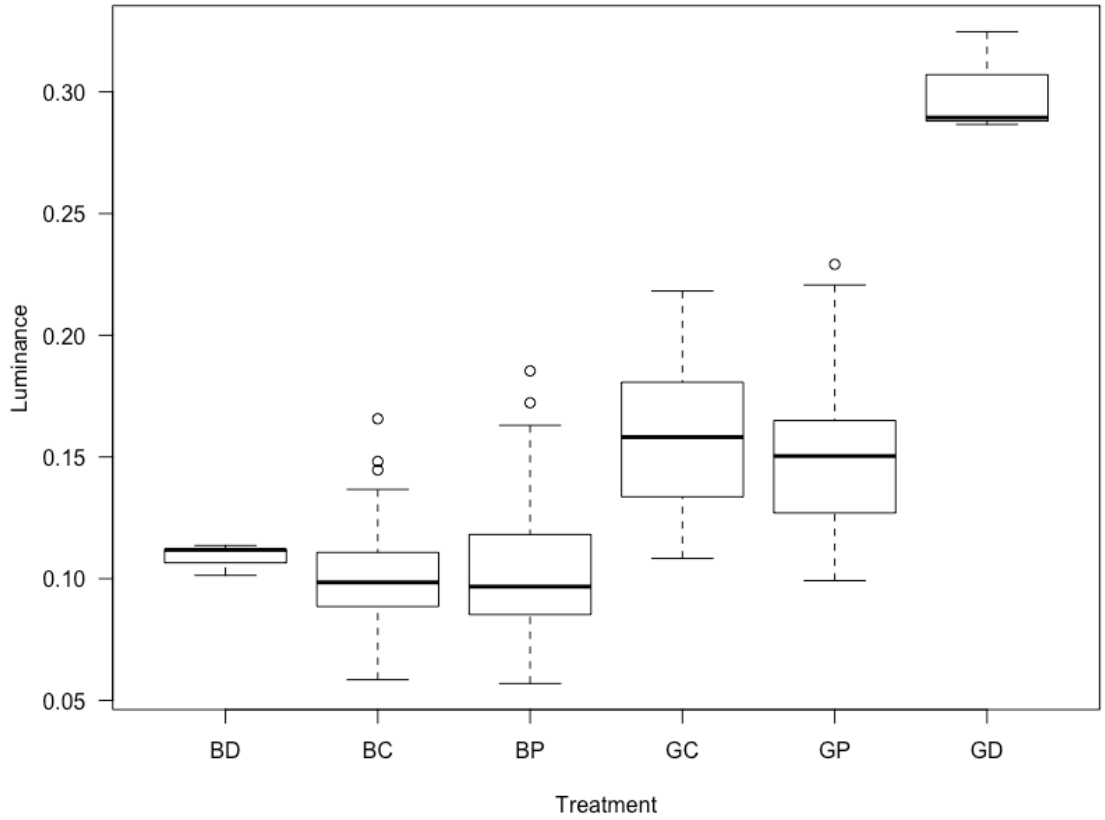


**Figure S3.3. Luminance response of *B. betularia* larvae to brown and green dowels.** Luminance of dowels and larvae calculated from dorsal double photon catches of a blue tit (*Cyanistes caeruleus*) under bright light conditions. Treatments are: BD, Brown dowel; BC, Brown control larvae; BP, Brown blindfolded larvae; GC, Green control larvae; GP, Green blindfolded larvae; GD, Green dowel.

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GAGAGCCCTCACGGCGGTCCGAGCGCGCTTTATGGCTATAAGTGCGTGGCTATACGCGGGCACCTTC
TCAGTAATCCCCGCGCTGGACTTCGGCTACGGCCACTATGTCCCCGAGGGGTACCTCACCAGCTGCA
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TTCCGTAAAGAATTTAAGAAACTTATGTTTAGGAGCAAAACTAAGCGCAAGCATGGAGGGACAATC
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GATGTAGAGGTAGTGGAGATGAGAGATATCCCGTTTCAGACTGATACCCTGCCTCGGATTGATGCTG
GGACGAACATTAGGACGATATCGTCACGAGTACCGGAACGGCAGGACTCACAGACGAGCATGAGC
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>Biston_arrestin1
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GACGAGGTCATGGGTCTCAACTTCTACAAGGAGCTGTTCCTCGCCTCCGAGCAGATCTACCCGCCGC
CGGAGAAACGGGACTACGAACTCACTAAGACGCAGGAGCGGCTGCTGAAGAAGCTGGGCGCGGGT
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AGGACGAGGGCGAGCCTTGCGGCGTGCACTACTACGTCAAGCTCTTCGTCGGCGACTCCGAGATCG
ACCGCTCGCACCGCAGGAGCACGGTGGCGCTGGGCGTCCGCAAGCTGCAGTTCGCGCCGTCGAAGC
CGGGCCCGCAGCCCTGCACCGTCGTCAGGAAGGACTTCGTGCTCTCCCCGGGGCAGCTGGAGCTGG
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ACCACAGCAACAAAGTGGTGAAGAAGGTCAAGGCCAGCATCCAGCAGGGCGTCGACGTCATGCTGT
TCCAGAGCGGGCAGTACCGGAACGTCGTCGCCAGCGTGGAGACGCAGGACGGGTGCCCGCTGCAGC
CCGGCTCGAGCCTGCAGAAGGTGCTGCACCTGACGCCGCTGCTGGCCTCCAACCGCGACAAGCGTG
GCATCGCGCTCGACGGCCAGCTGAAGCGCGCCGACACCACGCTCGCCTCCACCACGCTGCTTCTGGA
CGCGGAACAGCGCGACGCGTTCGGCATCGTAGTGAGCTACAGCGTCAAGGTAAAGCTGTACCTGGG
CGCGCTCAGTGGCGAGCTGGTCGCCGAGTTGCCTTTCATCCTGATGCATCCCAAGGAGGGGCGCGCC
AAGATGATCCACGCGGACAGCCAGGCCGACGTGGAGATGTTCCGCCAGGACACCGTGCACCACCAG
GAGAGCGTCGAGGTCTACTGA

> Biston_retinal_degeneration_B
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AATGAGCCTTATGAAGATGGACCTGGAGGTAAAGGTCAGTATACACAAAAGATTTATCATGTTGGC
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GGAATGCTTATCCATACACAAAAACCAGATACACCTGCCCATTTGTAGAGAAATTTTTACTAGAAAT
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GCCTACAAACTATGCAGAGTAGAATTTAGATATTGGGGCATGCAGACAAAATTAGAAAAATTTATT
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CCGCTGGGTCATAAAGCGGCACACTTAAACAATTTGATAAACGAACACGGCATCATTTTACACGCC
GCATACGGTTCCGGAAAAGATATAAGTGTTTATCACAACTGCGGGCTATCTCAGAGACAGATATAC
GCTATTGGGCGGATAAGTAAGAAGTATTGTAATATGGCGACGACTTTGAACGATGGTTACGCTTTGC
ACTTGGCTGAACTTAAGCAGCCGGGCGCAGTTCGGCCCGCTAGGGGCAATGCTCGTCTTCTAGTGCC
GCGGCGCCTTTTAGCTCCGGTCAACAACGTTGCTACTAATCGTGGCCGACGCTAA

**Figure S5.1.** Sequences of visual genes identified from the *B. betularia* genome

**Table S5.1.** Information on visual genes identified and measured in chapter 5

| Gene abbreviation | Gene full name | Proposed function | Exonic Structure |
|---|---|---|---|
| Arr | Arrestin-1 | Regulating the activity of G protein-coupled receptors (GPCRs) in the visual rhodopsin system (Wilden *et al.*, 1986). | 8 exons |
| RDB | Retinal degeneration B | Prevents photoreceptor degeneration in invertebrates (Hotta & Benzer, 1970). | 9 exons |
| UV | Ultraviolet | Provides spectral sensitivity in the UV wavelength range (Parry *et al.*, 2005; Briscoe, 2008). | 8 exons |
| BS1 | Blue splice variant 1 | Provides spectral sensitivity in the blue wavelength range (Parry *et al.*, 2005; Briscoe, 2008). | 8 exons |
| BS2 | Blue splice variant 2 | Unknown | 7 exons (skips $6^{th}$) |
| MelA | Melanopsin A | Non-visual photoresponses to light (Hattar *et al.*, 2002). | 3 exons (2, 3, 4) |
| MelB | Melanopsin B | Non-visual photoresponses to light (Hattar *et al.*, 2002). | 3 exons (1, 3, 4) |
| LW1 | Longwave 1 | Provides spectral sensitivity in the long (green-red) wavelength range (Parry *et al.*, 2005; Briscoe, 2008). | 8 exons |
| LW2 | Longwave 2 | Provides spectral sensitivity in the long (green-red) wavelength range (Arikawa, 2003). | 8 exons |

**Table S5.2** Cross information for *B. betularia* individuals used in gene quantification and electrophysiology experiments.

| **Experiment** | **Family ID #** | **Cross/parent information** | **N** |
|---|---|---|---|
| qPCR larvae | ♂/♀ 295 | *Carbonaria* ♀ x *carbonaria* ♂ | 4 |
| qPCR adults | ♂ 164 | *Typical* ♀ x *typical* ♂ | 4 |
| | ♂ 281 | *typical* ♀ x *typical* ♂ | |
| | ♀ 273 | *carbonaria* ♀ x *typical* ♂ | |
| | ♀ 277 | *carbonaria* ♀ x *carbonaria* ♂ | |
| Electrophysiology larvae | ♂/♀ 303 | *carbonaria* ♀ x *typical* ♂ | 5 |

**Table S5.3.** Details of primers described in methods for end-point PCR, qPCR, and sequencing reactions

| Primer(s) used | | Product length (bp) | Purpose |
|---|---|---|---|
| **Arrestin** | | | |
| Bb_Arr_68U21 AGCGGGACTTCGTCGATCACA | Bb_Arr_619L20 ACCGTCTCGCCGTGCATGTA | 571 | End-point PCR for presence/absence and sequencing |
| Bb_ArrQ_122U20 TGCTGGAGGAGGAGTACGTG | Bb_ArrQ_287L20 CCGCTCCTGCGTCTTAGTGA | 185 | qPCR |
| **Retinal Degeneration B** | | | |
| Bb_RDB_113U21 GTGGCGTGGAAATTCTTGTCA | Bb_RDB_687L21 TGTCTGTGGGCTCGGAGCATA | 595 | End point PCR for presence/absence and sequencing |
| Bb_RDB_113U21 GTGGCGTGGAAATTCTTGTCA | Bb_RDBQ_EB_429L23 CACCATAAAGTTGATCTTTGACT | 339 | qPCR |
| **Ultraviolet** | | | |
| Bbcon23183r: 555U ACACCGCACTAGCCCTACT | Bbcon23183r: 1077L GTGCGAACACCTGACAACCCAT | 542 | Verify exon 2 and 3 sequence; superpool |
| Bbcon128242r: 317U GGCCTCTAGACGGAAGATTATC | Bbcon128242r: 714L AAATGTGTCAAAACCACGTCACTG | 417 | Verify exon 4 and 5 sequence |
| Bbcon122456r: 161U ACTTCTCTCTTGGCGTGAAT | Bbcon122456r: 560L GCGAACACTTGTTTCACGATCC | 419 | Verify contig for exon 5 |
| Bbcon87111: 515U TGGAATCTCTTCGCGCTAATC | Bbcon87111: 911L CGACGATAGTTGTCCGGCACAT | 416 | Verify contig for exon 7 |
| Bbcon57760: 312U GGGTGTACGCAATCAACCAT | Bbcon57760: 831L GGCAACACTGGTGGTGGTAGAG | 359 | Verify exon 7 and 8 |
| Bbcon23183r:125L TGCGGTATGCTGCCACTTGTG | | - | BAC sequencing primer to obtain exon 1 |
| Bbcon23183r:517L GCCGGGTACGATAGCCAGTGC | | - | BAC sequencing primer to obtain exon 2 |
| Bbcon23183r: 1023U AAAGCGCCGATATTCATCTAT | | - | Sequencing primer to determine exon 4 |
| Bbcon87111:981U GCACAAGAGTTCGCCTATAC | | - | BAC sequencing primer in intron 6 to reach exon 7 |
| Bbcon57760r:290L ACGGATCTATGCAAGACACTAC | | - | BAC sequencing primer to determine exon 6 |

| | | | |
|---|---|---|---|
| Bb_UVRh_exon7_F CCTGGGTCTACGCCATTAGTCAT | | - | BAC sequencing primer |
| Bbcon57760r: 667U TAAGTCCTCCCTTTGTAAATG | | - | BAC sequencing primer to confirm exon 8 and beyond |
| Bb_UVRh_1000U CGGGATTATCCGATCATCC TGTT | Bbcon23193r_517L GCCGGGTACGATAGCCA GTGC | ~277 | PCR and sequence to clarify where exon 2 begins |
| Bb_UVRh_1000U CGGGATTATCCGATCATCC TGTT | Bbcon23183r1077L GTGCGAACACCTGACAA CCCAT | ~525 | PCR and sequence past exon 2 |
| Bb_UVRh_960U CGCTGTGGCGTGTAAAGC TGT | Bb_UVRh_1112L ATTATTTCATGCTGGCGC GTTTG | 175 | To target UV opsin gene specifically. Used for qPCR temperature gradient |
| Bb_UVRh_32U GCGCTCATTTTGCAGCTCT CA nested: Bbcon23183r_517L GCCGGGTACGATAGCCAG TGC | Bbcon122456r560L GCGAACACTTGTTTCACG ATCC Nested: Bbcon23183r_1077L GTGCGAACACCTGACAA CCCAT | 709 | Sequence prediction validation; PCR then sequencing with nested primers |
| Bb_UVRh_32U GCGCTCATTTTGCAGCTCT CA | Bbcon23183r_517L GCCGGGTACGATAGCCA GTGC | 115 | Sequence prediction validation; PCR then sequencing |
| Bb_UVRh_32U GCGCTCATTTTGCAGCTCT CA | Bbcon23183r1077L GCCGGGTACGATAGCCA GTGC Nested: Bbcon23183r_517L GCCGGGTACGATAGCCA GTGC | 363 | Sequence prediction validation; PCR then sequencing with nested primers |
| Bb_UVRh_1000U CGGGATTATCCGATCATCC TGTT | Bbcon122456r560L GCGAACACTTGTTTCACG ATCC (nested: Bbcon23183r_517L &1077L) | 870 | Sequence prediction validation; PCR then sequencing with nested primers |
| Bb_UVRh_1000U CGGGATTATCCGATCATCC TGTT | Bbcon23193r_517L GCCGGGTACGATAGCCA GTGC | 276 | Sequence prediction validation; PCR then sequencing |
| Bbcon12842r317U GGCCTCTAGACGGAAGAT TATC Nested: Bb_UVRh_960U CGCTGTGGCGTGTAAAGC TGT | Bb_UVRh_1401L GCAAGCCACAGTCGTCGT TTAACAATAC Nested: Bb_UVRh_1112L ATTATTTCATGCTGGCGC GTTTG | 956 | Sequence prediction validation; PCR then sequencing with nested primers |
| Bb_UVRh_375U GGGTTGTCAGGTGTTCGCA CTTA | Bb_UVRh_796L TCCGCGCCAGAACTTTGA TTAGC | 444 | To target UV opsin splice variant 1 specifically. Used for qPCR temperature gradient |
| Bb_UVRh_238U TTTTCTACACATTCAACAA TC | Bb_UVRh_650L CGAGGCAACGAACAGGA AAC | 432 | To target UV opsin splice variant 2 specifically. Used for qPCR temperature gradient |
| **Blue (BS1 and BS2)** | | | |

| | | | |
|---|---|---|---|
| Bbcon47669r: 495U CTTCCCGGCCGTTAGCAAGTA | Bbcon47669r: 900L TCCCGCCAAAACTTCCAACTGT | 427 | Verify exon 2 |
| Bbcon57760r: 313U GGTGTACGCAATCAACCATC | Bbcon57760r: 831L GGCAACACTGGTGGTGGTAGAG | 540 | Verify exon 7 and 8 |
| Bbcon05474r: 683U GGACGATTTGTGCCAGGTAAAC | Bbcon05474r: 1036L CATTCGCGAGCACAATAC | 371 | Verify exon 4 to check correspondence of BAC with exon 2 and 8; superpool |
| Bbcon47669r: 478L GGAAGTTGCGCCAGTGCTCGTG | | - | BAC sequencing primer to obtain exon 1 |
| Bbcon57760r: 313U GGTGTACGCAATCAACCATC | | - | BAC sequencing primer to obtain 3' UTR |
| Bb_blueOps_96L CGGGGTTTGATTGATGTTACTAGGT | | - | BAC sequencing primer |
| Bb_blueOps_795U AGCCAACAAGGAAGACGCAAGCA | Bb_blueOps_1090L TGGCAACACTGGTGGTGGTAGA | 317 | To target blue opsin gene specifically. Used for qPCR temperature gradient |
| Bb_blueOps_293U TCCGGCACAAGTGAGAAGCGACT<br>Nested: Bbcon47669r: 495U CTTCCCGGCCGTTAGCAAGTA | Bb_blueOps_1021L ATCCCTCTGGCACAAATCGTCCC<br>Nested: Bbcon47669r: 478L GGAAGTTGCGCCAGTGCTCGTG | 751 | Sequence prediction validation; PCR then sequencing with nested primers |
| Bb_blueOps_711U AAGCCAAGAAGATGAACGTGAAGT | Bb_blueOps_1134L TCGAACCTTGTTAATGGACCTTGT | 447 | Sequence prediction validation; PCR then sequencing |
| Bb_blueOps_728U GCGCTGTCGCTTCTGACG | Bb_blueOps_1090L TGGCAACACTGGTGGTGGTAGA | 385 | To target blue opsin splice variant 2 specifically. Used for qPCR temperature gradient |
| Bb_blueOps_505U GCTGGTCTGCTGATAGCCTTCAC | Bb_blueOps_718L CGTCAGAAGCGACTTTTTTGAACAGTTG | 236 | To target blue opsin splice variant 2 specifically. Used for qPCR temperature gradient |
| **Melanopsin (A and B)** | | | |
| Bb_mopsinX_84U TCGCAGAAGATGCTGGGAGTGCT | Bb_mopsinX_671L TGCTCAGAACTCATCCGCTTATC | 610 | Superpool and matrix pool for BAC |
| Bb_mopsinA_186U ACTCCTGGCAACATCCTGATAGC | Bb_mopsinA_306L ACCGCCTACAAATCCATATACGAC | 592 | Superpool and matrix pool for BAC |
| Bb_mopsinA_188U TGAGACTTTGGAAGCGACGAAGA | Bb_mopsinA_368L AAACAGCCCATATTTCCGCCACA | 203 | To target gene melanopsin A specifically. Used for qPCR temperature gradient |
| Bb_mopsinB_211U TACCGCCTCGCTGAATCTGATGA | Bb_mopsinB_427L CCACTGTAGCGAAGAGTAGATAGAGCAC | 244 | To target gene melanopsin B specifically. Used for |

| | | | |
|---|---|---|---|
| | | | qPCR temperature gradient |
| Bb_mopsinB_1064U AATAATCAGGCCCATTCA GACGAC | Bb_mopsinA_306L ACCGCCTACAAATCCATA TACGAC Nested: Bb_mopsinA_1340L TTCGTATGGTCTCCAGCG GTATA | 989 | Sequence prediction validation; PCR then sequencing with nested primers |
| Bb_mopsinB_1169U GTCAAGAGGAACCAATCA TCGTAATAAG | Bb_mopsin_1918L GAGCTAGGTTGGCTATCA GGATGTT Nested: Bb_mopsinA_1340L TTCGTATGGTCTCCAGCG GTATA | 774 | Sequence prediction validation; PCR then sequencing with nested primers |
| Bb_mopsinA_946U GTGACGTGTAACACATGC GATGTC | Bb_mopsinA_306L ACCGCCTACAAATCCATA TACGAC Nested: Bb_mopsinA_1340L TTCGTATGGTCTCCAGCG GTATA | 956 | Sequence prediction validation; PCR then sequencing with nested primers |
| **Long wavelength 1** | | | |
| Bbcon19523r: 1149U AGCTGCCAACCAAACCGT CGTC | Bbcon19523r: 1695L TAAATAACCATGCCGTTG C | 526 | To confirm exon 1 and 2 sequence in gene copy 1; superpool |
| Bbcon03634: 1632U GGGCGTCTTCGAGAGCAT GA | Bbcon03634: 2255L AGCGCCTGCTGGTACTTA GGAT | 643 | To confirm exon 7 and 8 sequence in gene copy 1; superpool |
| Bbcon19523r: 1184L TCTATCATATGGAGCATATCTG | | - | BAC sequencing primer to obtain sequence before exon 1 in gene copy 1 |
| Bbcon03634: 2247U TTCCAGCCATCCTAAGTACC | | - | BAC sequencing primer to obtain sequence after exon 8 in gene copy 1 |
| Bb_LW1_1382U ACTAGCGCAGAGTGCAAA CTAGC | Bb_LW1_1717L CTGTGGTCGTTCGTCTTG AGATT | 358 | To target long wavelength copy 1 opsin gene specifically. Used for qPCR temperature gradient |
| Bb_LW1_519U TGAGAGGTGTATGTTCGC AGGTT | Bb_LW1_747L GATGAAGCCAAGAACGC CGATAG | 251 | Sequence prediction validation; PCR then sequencing |
| **Long wavelength 2** | | | |
| Bbcon08719: 2040U GGTCAAGTGGCCACCTAC TCC | Bbcon08719: 2612L AGCCGAGAACACCGATA GTGAA | 592 | To confirm exon 1 and 2 sequence in gene copy 2; superpool |
| Bbcon34441r: 377U TACGCTGGAATCTTCGAG AGCA | Bbcon34441r: 811L TAGCGCCTTCTGGTATCT A | 454 | To confirm exon 7 and 8 sequence in gene copy 2; superpool |

| | | | |
|---|---|---|---|
| Bbcon34441: 814U ATACCAGAAGGCGCTATATG | | - | BAC sequencing primer to obtain sequence after exon 8 in gene copy 2 |
| Bb_LW2_452U AGAAACCAGTAAACATGT CACCTTCC | Bb_LW2_882L TGGTCCAGATAGAAACG CAACCG | 453 | PCR and then sequencing to find missing exon 3 and to rectify error within exon 1 from previous *Biston* sequence |
| Bbcon08719: 2078L CAGCATGTCCGAAGGGACCTTG | | - | BAC sequencing primer to obtain sequence before exon 1 in gene copy 2 |
| Bbcon34441r_377U TACGCTGGAATCTTCGAG AGCA | Bb_LW2_1755L TCGCAAGCGGTCGTCATA GTTGT | 406 | To target long wavelength copy 2 opsin gene specifically. Used for qPCR temperature gradient. Sequencing validation |
| **Control genes** | | | |
| T7 promotor primer TAATACGACTCACTATAGGG | | - | Was used as fosmid forward sequencing primer instead of FosmidF, as provided larger insert |
| FosmR (fosmidR) CTCGTATGTTGTGTGGAATTGTGAGC | | - | Was used as fosmid reverse sequencing primer in BAC sequencing |
| Bb_Rps3A_93U CCGCAAAGACTGGTACGA TGT | Bb_Rps3A_279L TGCACGTCCTCGGCGATC AAA | 207 | Control gene for qPCR. Optimised using temperature gradient |
| Bb_spectrin_278U GCGCTGAAGGAGTTCTCG ATGAT | Bb_spectrin_705L TGGAATAGCGTGCGCGT GAAGT | 449 | Control gene for qPCR. Optimised using temperature gradient |

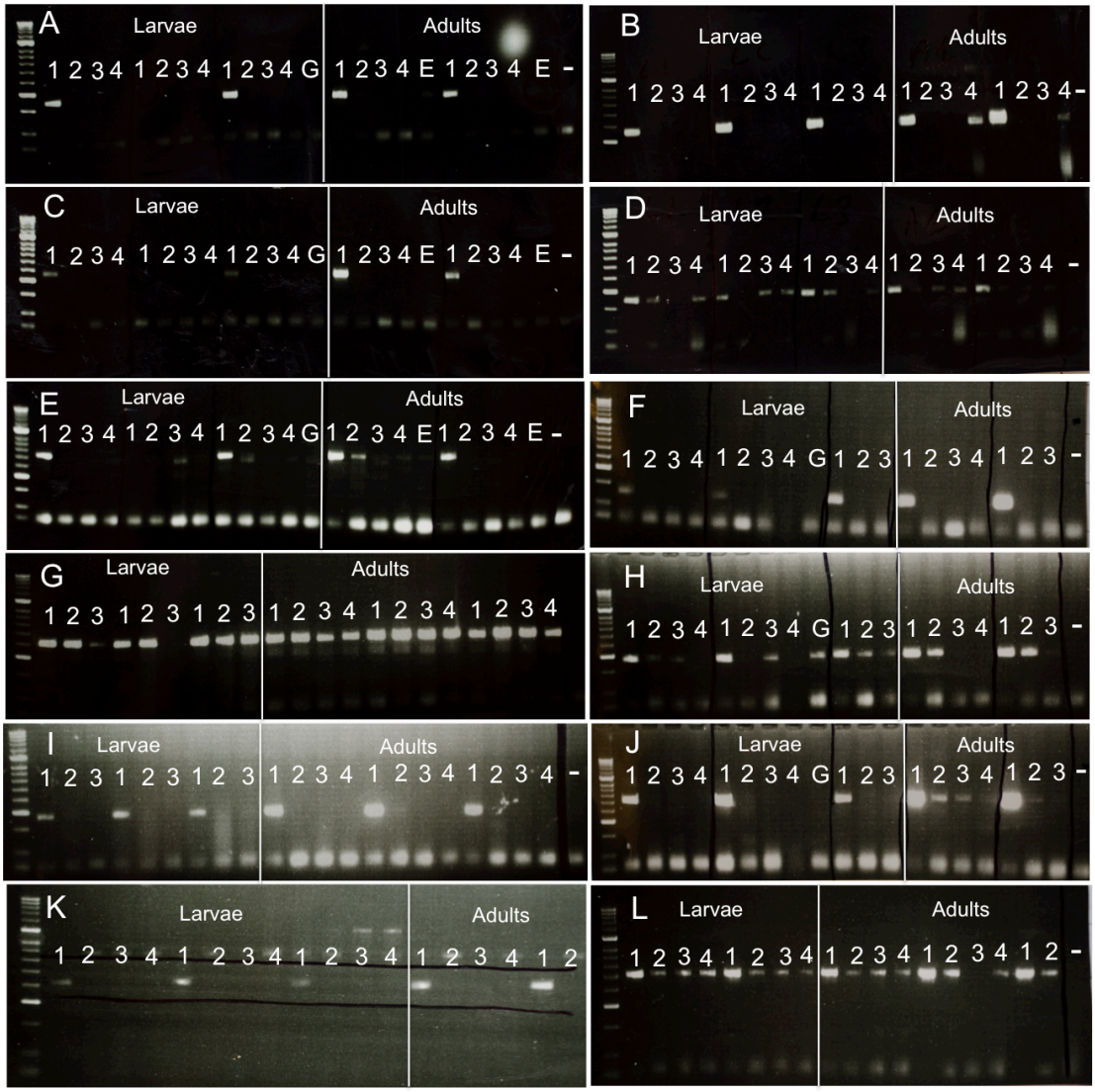


**Figure S6.1. End-point PCR gel images showing opsin gene expression in tissues of larval and adult stage Lepidoptera.** (A) *S. dentaria* UV; (B) *A. grossulariata* UV; (C) *S. dentaria* blue; (D) *A. grossulariata* blue; (E) *S. dentaria* LW2; (F) *A. caja* UV; (G) *A. psi* UV; (H) *A. caja* blue; (I) *A. psi* blue; (J) *A. caja* LW1; (K) *O. plecta* UV; (L) *O. plecta* LW1. Numbers and letters indicate tissue types, where 1= head; 2= thorax; 3=abdomen; 4= claspers in larvae, genitalia dermis in adults; E= eggs; G=gonads; - = negative control. 5µL of PCR product loaded onto 2% agarose gel stained with 1% ethidum bromide, run under 175V, 500A, and visualised under UV light.

**Table S6.1.** Information on degenerate primers used to identify presence or absence of opsin genes in Lepidoptera.

| Gene | Species | Forward primer | Reverse primer | Primer design |
|---|---|---|---|---|
| Control | All Lepidoptera | Dg_lep_RpS8 _3U: GGGTATYAGYCGBGATC AYTG | Dg_lep_RpS8 _600L: GCCCTCTTRGAYTTRATC TTY | Degenerate |
| UV | *Laothoe populi* | Dg_BOM_UV_569U: GGGGCAGATAYGTWCCWGAG GG | Dg_BOM_UV_868L: CCRTAKGGTGTCCABGABGC | Degenerate |
| | *Bombyx mori* | Bm_UV_47U: GACTTAGCAGCCGTACCAGA | Bm_UV_1017L: TTGCGGTCGAAGTTGTATCAT | Genome |
| | *Phlogophora meticulosa, Agrotis exclamationis, Ochropleura plecta, Diarsia mendica, Euplexia lucipara* | Dg_NOC_UV_ 79U20(2): ATGCTGGGCGMDGGVCTGAC | Dg_NOC_UV_464L19(3): TCTARRGGBCGRGTGATTG | Degenerate |
| | *Helicoverpa armigera* | Ha_UV_34U: GCGCACTTTGCAGCATACA | Ha_UV_1039L: ACCATGGCATGCGACGTTG | Genome |
| | *Acronicta psi* | Dg_NOC_UV_ 79U20(1): TGCTCGGCGMDGGVCTCAC | Dg_NOC_UV_ 464L19(1): TCGARRGGBCGRGTGATTG | Degenerate |
| | *Euproctis similis, Arctia caja* | Dg_NOC_UV_ 864U21: CYTRTTYGTRGCRTCRTGGAC | Dg_NOC_UV_ 1000L23(1): TTMGGRTGRCTGATKGCATAMA C | Degenerate |
| | *Phigalia pilosaria* | Pp_uvops_94U: GCACACACCGCTCTCGCACTT | Pp_uvops_981L: CTGAGTCGTCGGGCTCGTC | Genome |
| | *Biston betularia* | Bbcon23183r_555U: ACACCGCACTAGCCCTACT | Bbcon122456r_560L: GCGAACACTTGTTTCACGATCC | Genome |
| | *Odontopera bidentata* | Ob_uvops_52U: AAGGCCGGAGATGTAGAGAT G | Ob_uvops_1043L: AGGTTCGTCGATCTGTAGCCA | Genome |
| | *Ourapteryx sambucaria, Abraxas grossulariata, Selenia dentaria* | Dg_GEO_UV_ 372U19: YCAGGTGTTYGCACTBATG | Dg_GEO_UV_ 701L22: TTHACGATBCCRCTGTAGAAGT | Degenerate |
| | *Operophtera brumata* | Op.b_UVops_164U20: CTCTGGCGCTCCTTTACACA | Op.b_UVops_ 1095L21: GCGCTGCTGTTGACAGTATTG | Genome |
| | *Plodia interpunctella* | Pi_uvops_4U: GGCCGGTGATGTAGAGTTGTT | Pi_uvops_1040L: CGGCCCGCTGACTGTATTAGT | Genome |
| | *Ephestia elutella* | Dg_PYR_UVops_758U23: RGAACARGCTAARAARATGA AC | Dg_PYR_UVops_1048L21: GTYGATYTGSAGCCATGGCAT | Degenerate |
| | *Galleria mellonella* | Pi_uvops_4U: GGCCGGTGATGTAGAGTTGTT | Pi_uvops_1040L: CGGCCCGCTGACTGTATTAGT | Genome |
| | *Bicyclus anynana* | Ba_uvops_121U: CCCGACCACTGGATGTC ATAC | Ba_uvops_1079L: GGTGCCAGTCGATGCGTTGT | Genome |
| **Blue** | *Laothoe populi and eyed hawk* | Dg_BOM_B_100U: AACATYCCAGARGAACATCA AGAC | Dg_BOM_B_673L: ATRCAGTARCTCCACACGAAGA T | Degenerate |
| | *Bombyx mori* | Bm_blue_27U: AAGCGACATCGGACCAATG | Bm_blue_1101L: TACCGACGCTGGTTGTTGTG | Genome |
| | *Phlogophora meticulosa, Agrotis exclamationis, Euplexia lucipara, Ochropleura plecta, Acronicta psi, Euproctis similis, Arctia caja* | Dg_NOC_B_763U: CAAGCCAARAAGATGAACGTS AA | Dg_NOC_B_1012L: TCCGCCCTGTATCTGGGATGATT | Degenerate |
| | *Diarsia mendica* | Dg_NOC_B_217U22: AACGGCATHGTCATMTGGAT WT | Dg_NOC_B_607L23: GTRAAGTAGTCRAASGARCASG T | Degenerate |
| | *Helicoverpa armigera* | Ha_blue_60U: CAAAGAGGTGGTCGAGCACA T | Ha_blue_1118L: GCGGGTTGGGCAGTAGACT | Genome |

| | | | | |
|---|---|---|---|---|
| | *Phigalia pilosaria* | Pp_blueops_1U: ATGGCGTTTAATTTCACCGAC | Pp_blueops_1020L: GAGCTCCGCCCTGTATCTA | Genome |
| | *Biston betularia* | Bbcon47669r_495U: CTTCCCGGCCGTTAGCAAGTA | Bbcon57760r_ 831L: GGCAACACTGGTGGTGGTAGAG | Genome |
| | *Odontopera bidentata* | Ob_blueops_32U: CCATGGCGTACCCACTGAAAT | Ob_blueops_1020L: TGGAGCTCCGCCCTGTATCTT | Genome |
| | *Ourapteryx sambucaria, Abraxas grossulariata, Selenia dentaria* | Dg_NG_blue_763U: CAAGCCAARAAGATGAACG | Dg_NG_blue_979L: CAYGGRTCTATGCARGABAC | Degenerate |
| | *Operophtera brumata* | Op.b_blueops_132U21: GAGGAGTTTCCCAGCCGTGAG | Op.b_blueops_651L21: ACAAAGATGCACGCCACGAAC | Genome |
| | *Plodia interpunctella* | Pi_blueops_67U: GCACGACCACTGGCGCAACTT | Pi_blueops_1006L: ATCTTGTTCGCGGACTCCC | Genome |
| | *Galleria mellonella, Ephestia elutella* | Dg_PYR_Blue ops_137U: GCAACTTCCCCGCAGTCAGCAA | Dg_PYR_Blue ops_648L: GATGCAGGCSACRAACACCTTC | Degenerate |
| | *Bicyclus anynana* | Ba_blueops_50U: AAATGGTCTCACAAGAAGTGG | Ba_blueops_1081L: ACTGGAGGTGGATACGCTGTC | Genome |
| **LW1** | *Laothoe populi* | Dg_BOM_LW_741U: GGCCGTAGCTGCYCACGARA | Dg_BOM_LW_971L: RGCGTTAGCYTTGGCRAAGA | Degenerate |
| | *Bombyx mori* | Bm_LW_70U: GGAGCCGCTAACCAAACCGTT | Bm_LW_1103L: AGACAGCTGTGGCACCCGAAG | Genome |
| | *Phlogophora meticulosa, Agrotis exclamationis, small angle shades, Ochropleura plecta, Euplexia lucipara, Diarsia mendica, Arctia caja* | Dg_NOC_LW_363U: CGAAACRTGGGTHTGGGGTCCT | Dg_NOC_LW_835L: GCHACCTTHGCYAATTTGCACTC | Genome |
| | *Helicoverpa armigera* | Ha_LW1_43U: CAAGCATGGGGCGGCCAGGTA | Ha_LW1_1036L: ATCTCTGGTACAACGCAGC | Genome |
| | *Acronicta psi* | Dg_NP_LW_226U: GGCAAYGGMATGGTYATCTA | Dg_NP_LW_649L: TARCTKCGGCTGAACCARTC | Degenerate |
| | *Euproctis similis* | Dg_NP_LW_226U: GGCAAYGGMATGGTYATCTA | Dg_NOC_LW_835L: GCHACCTTHGCYAATTTGCACTC | Degenerate |
| | *Phigalia pilosaria* | Pp_lw1ops_71U: CCTACGGAGCTGCCAACAAA | Pp_lw1ops_1056L: TGCACGCCAACGAAGGGAAC | Genome |
| | *Biston betularia* | Bbcon19523r_1149UL: AGCTGCCAACCAAACCGTCGTC | Bbcon03634_2255L: AGCGCCTGCTGGTACTTAGGAT | Genome |
| | *Odontopera bidentata* | Ob_lw1ops_70U: GCGTACGGAGCCTCCAATCAA | Ob_lw1ops_1121L: GGGCTTTTCTTCGGAGACTG | Genome |
| | *Ourapteryx sambucaria, Abraxas grossulariata, Selenia dentaria* | Dg_GB_LW1_160U: ATGAACCCWCTWTGGCAY | Dg_GB_LW1_322L: GGRGACATVGCRCACATCAT | Degenerate |
| | *Operophtera brumata* | Op.b_LWops_58U20: GGTCAAGTGGCAGCATACGG | Op.b_LWops_ 966L21: GCTTTCGCGAAGAGTGATCCC | Genome |
| | *Plodia interpunctella* | Pi_lwops_106U: GTGCCACCAGAATTGCTACA | Pi_lwops_1061L: GCCTGGCATGATAGCGATG | Genome |
| | *Ephestia elutella* | Gm_LWops_76U21: ATCACTGGCAACGGAATGGTC | Gm_LWops_672L21: GCACTCGTGTTAGCCGCTTCA | Genome |
| | *Galleria mellonella* | Gm_LWops_76U21: ATCACTGGCAACGGAATGGTC | Gm_LWops_672L21: GCACTCGTGTTAGCCGCTTCA | Genome |
| | *Bicyclus anynana* | Ba_lwops_34U: ATCGCAGCTCTGCAAGCATGG | Ba_lwops_1007L: TCGGGTGGCTGATACCGTATA | Genome |
| | *Helicoverpa armigera* | Ha_LW2_73U: AACCAGACCGTCGTGGACAAA | Ha_LW2_1030L: GGGGAACTTCTGGTACAACAC | Genome |
| | *Phigalia pilosaria* | Pp_lw2ops_58U: TACTCCAACCAAACCGTCGTC | Pp_lw2ops_1093L: CTCATCAGAGATAGCCGTGAC | Genome |

| | | | | |
|---|---|---|---|---|
| **LW2** | *Biston betularia* | Bbcon08719_2040U: GGTCAAGTGGCCACCTACTCC | Bbcon34441r_811L: TAGCGCCTTCTGGTATCTA | Genome |
| | *Odontopera bidentata* | Ob_lw2ops_55U: GGCGCACGGAACCTCTAACC | Ob_lw2ops_1021L: CTCTTGTACAAAGCCGCTTGA | Genome |
| | *Ourapteryx sambucaria, Abraxas grossulariata, Selenia dentaria* | Dg_geo_LW2 _370U: GGTCCATTYGCDTGTGAACT | Dg_geo_LW2 _866L: GTCCASGCCATGAACCAYA | Degenerate |

**Table S6.2.** Statistical output from phylogenetic signal analysis in opsin genes across Lepidoptera (significant results in red).

| **Stage** | **Gene** | **Test statistic (*D*)** | ***P*-value (significantly differs from random?)** |
|---|---|---|---|
| Larvae | Blue | 0.58 | 0.11 *NS* |
| | UV | 0.55 | 0.18 *NS* |
| | LW1 | 0.39 | 0.03 ** |
| | LW2 | 0.46 | 0.10 *NS* |
| Adults | Blue | 0.76 | 0.22 *NS* |
| | UV | 1.08 | 0.54 *NS* |
| | LW1 | 0.95 | 0.40 *NS* |
| | LW2 | 0.01 | 0.01 ** |
| **Stage** | | **Test statistic (*K*)** | ***P*-value (significantly differs from random?)** |
| Larvae | | 0.18 | 0.18 |
| Adults | | 0.17 | 0.22 |

**Table S6.3.** Statistical output from GLM analysis testing life-history predictors upon opsin expression across Lepidoptera (significant results in red).

| **Predictor** | **Residuals deviance** | **Z value** | ***P*-value** |
|---|---|---|---|
| Stage | 3.616 | -5.95 | <0.0001 **** |
| Tissue | 0.820 | -0.70 | 0.50 *NS* |
| Sex | 19.021 | -2.50 | 0.01 * |
| Gene | 90.290 | 0.80 | 0.02 * |
| Polyphagy | 6.812 | -0.75 | 0.45200 *NS* |
| Colour | 108.384 | 2.00 | <0.001 *** |

**Table S6.4.** Source of visual gene sequences used for phylogenies shown in Fig. 5.3, including *Biston betularia*, with Genbank accession numbers where available.

| Species | Description | Source | Accession number |
|---|---|---|---|
| **Ultraviolet (UV)** | | | |
| *Biston betularia* | Isoform A | Genome – verified | MH166324 |
| | Isoform B | | MH166325 |
| *Odontopera bidentata* | | Genome- predicted | - |
| *Operophtera brumata* | | Genome – predicted | - |
| *Helicoverpa armigera* | | NCBI BLAST | HQ641391.1 |
| *Agrotis ipsilon* | | NCBI BLAST | KF539451.1 |
| *Agrotis segetum* | | NCBI BLAST | KF539450.1 |
| *Ctenoplusia agnata* | | NCBI BLAST | KF539452.1 |
| *Mythimna separata* | | NCBI BLAST | KF539458.1 |
| *Spodoptera exigua* | | NCBI BLAST | KF539459.1 |
| *Spodoptera litura* | | NCBI BLAST | KF539460.1 |
| *Chilo suppressalis* | | NCBI BLAST | KF539453.1 |
| *Loxostege sticticalis* | | NCBI BLAST | KF539455.1 |
| *Manduca sexta* | Manop 2 | NCBI BLAST | L78081.1 |
| *Papilio glaucus* | | NCBI BLAST | AF077191.1 |
| *Danaus plexippus* | | NCBI BLAST | AY605546.1 |
| *Vanessa cardui* | | NCBI BLAST | AF414074.2 |
| *Apis mellifera* | | NCBI search | BK005513.1 |
| **Blue (Bl)** | | | |
| *Biston betularia* | Isoform A | Genome- verified | MH166326 |
| | Isoform B | | MH166327 |
| *Phigalia pilosaria* | | Genome- predicted | - |
| *Odontopera bidentata* | | Genome- predicted | - |
| *Helicoverpa armigera* | | NCBI BLAST | JX644013.1 |
| *Agrotis ipsilon* | | NCBI BLAST | KF539430.1 |
| *Agrotis segetum* | | NCBI BLAST | KF539429.1 |
| *Ctenoplusia agnata* | | NCBI BLAST | KF539431.1 |
| *Mythimna separata* | | NCBI BLAST | KF539428.1 |
| *Spodoptera exigua* | | NCBI BLAST | KF539436.1 |
| *Spodoptera litura* | | NCBI BLAST | KF539437.1 |
| *Chilo suppressalis* | | NCBI BLAST | KF539432.1 |
| *Loxostege sticticalis* | | NCBI BLAST | KF539434.1 |
| *Plodia interpunctella* | | Genome- predicted | - |
| *Plutella xylostella* | | NCBI BLAST | NM_001305481.1 |
| *Manduca sexta* | Manop 3 | NCBI BLAST | AD001674.1 |
| *Apis mellifera* | | NCBI | BK005512.1 |
| **Long wavelength copy one (LW1)** | | | |
| *Biston betularia* | | Genome- verified | MH166328 |
| *Phigalia pilosaria* | | Genome- predicted | - |
| *Odontopera bidentata* | | Genome- predicted | - |
| *Helicoverpa armigera* | | NCBI BLAST | JX392054.1 |
| *Agrotis ipsilon* | | NCBI BLAST | KF539439.1 |
| *Agrotis segetum* | | NCBI BLAST | KF539438.1 |
| *Ctenoplusia agnata* | | NCBI BLAST | KF539440.1 |
| *Spodoptera exigua* | | NCBI BLAST | KF539448.1 |
| *Spodoptera litura* | | NCBI BLAST | KF539449.1 |
| *Chilo suppressalis* | | NCBI BLAST | KF539441.1 |
| *Loxostege sticticalis* | | NCBI BLAST | KF539443.1 |
| *Plodia interpunctella* | | Genome- predicted | - |
| *Manduca sexta* | Manop1 | NCBI BLAST | L78080.1 |
| *Bombyx mori* | | NCBI BLAST | XM_021349577.1 |
| *Macroglossum stellatarum* | | NCBI BLAST | KF539444.1 |
| *Papilio glaucus* | | NCBI BLAST | AF077189.1 |
| *Danaus plexippus* | | NCBI BLAST | AY605545.1 |
| *Bicyclus anynana* | | NCBI BLAST | Y918895.2 |
| *Vanessa cardui* | | NCBI BLAST | AF385333.2 |
| ***Long wavelength copy two (LW2)*** | | | |
| *Biston betularia* | | Genome- verified | MH166329 |
| *Phigalia pilosaria* | | Genome- predicted | - |
| *Odontopera bidentata* | | Genome- predicted | - |
| *Helicoverpa armigera* | | NCBI BLAST | KJ010188.1 |

| | | | |
|---|---|---|---|
| *Papilio glaucus* | | NCBI BLAST | AF077190.1 |
| *Apis mellifera* | | NCBI search | NM_001011639.2 |
| **Melanopsin (Mel)** | | | |
| *Biston betularia* | Isoform A | Genome- verified | MH166330 |
| | Isoform B | | MH166331 |
| **Arrestin-1 (Arr-1)** | | | |
| *Biston betularia* | | Genome- verified | MH166332 |
| *Bombyx mori* | | NCBI BLAST | XM_004925776.3 |
| *Helicoverpa armigera* | | NCBI BLAST | XM_021342135.1 |
| *Spodoptera litura* | | NCBI BLAST | XM_022965205.1 |
| *Amyelois transitella* | | NCBI BLAST | XM_013338646.1 |
| *Papilio machaon* | | NCBI BLAST | XM_014513530.1 |
| *Papilio polytes* | | NCBI BLAST | XM_013280508.1 |
| *Papilio xuthus* | | NCBI BLAST | XM_013323333.1 |
| *Pieris rapae* | | NCBI BLAST | XM_022262271.1 |
| *Apis mellifera* | | NCBI BLAST | XM_016916562.1 |
| *Drosophila melanogaster* | Protein for tblastn | NCBI search | NP_476681 |
| **Retinal degeneration B** | | | |
| *Biston betularia* | | Genome- verified | MH166333 |
| *Bombyx mori* | | NCBI BLAST | XM_004929426.3 |
| *Helicoverpa armigera* | | NCBI BLAST | XM_021326572.1 |
| *Spodoptera litura* | | NCBI BLAST | XM_022958969.1 |
| *Plutella xylostella* | | NCBI BLAST | XM_011556250.1 |
| *Amyelois transitella* | | NCBI BLAST | XM_013327659.1 |
| *Papilio machaon* | | NCBI BLAST | XM_014508247.1 |
| *Papilio polytes* | | NCBI BLAST | XM_013287007.1 |
| *Papilio xuthus* | | NCBI BLAST | XM_013324421.1 |
| *Pieris rapae* | | NCBI BLAST | XM_022257986.1 |
| *Bicyclus anynana* | | NCBI BLAST | XM_024080572.1 |
| *Apis mellifera* | | NCBI BLAST | XM_016911166.1 |
| *Drosophila melanogaster* | Protein for tblastn | NCBI search | NP_476788 |
| **Control gene (spectrin)** | | | |
| *Biston betularia* | Reference gene for qPCR | NCBI search | KT182638 |

**Figure S7.1.** Example of larval colouration in final instar *B. betularia* reared on striped green and brown dowels.